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Developing a universal T cell for use in adoptive immunotherapy

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

I, Benjamin David Grimshaw, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Adoptive immunotherapy with genetically-engineered T-cells is showing promise in a clinical setting. However, this approach is limited by the requirement to generate autologous, patient-derived, T-cells, which is both costly and time-consuming. To address this, a methodology which would allow the generation of allogeneic universal T-cells would be highly beneficial. In this project, I have attempted to develop a protocol by which the following T-cell modifications will be introduced: (1) a chimeric antigen receptor (CAR), (2) a sort-suicide gene, (3) human leukocyte antigen (HLA) knock-down, (4) T cell receptor (TCR) knock-down (5) natural killer (NK) cell inhibition. Various strategies have been tested to attempt these modifications. Firstly, I knocked down HLA Class I by using two viral proteins, US11 and infected cell protein (ICP)-47, which give knock-down of 88% and 91% of HLA Class I expression respectively when tested in donor peripheral blood mononuclear cells (PBMCs). In a mixed lymphocyte reaction (MLR) with HLA mis-matched donors, US11 has clearly been shown to reduce the proliferation of effector PBMCs. To address rejection of HLA negative cells by NK cells, HLA-G has been cloned and expressed in cell lines and donor PBMCs to mimic the expression of HLA-G by trophoblast cells in the uterus and by certain cancers. TCR knock-down has been demonstrated in PBMCs, and CD52 knock-down has been achieved in a cell line by using DNA-editing transcription activator-like effector nucleases (TALENs). A CAR can be introduced to redirect the T-cells and a sort-suicide gene can be used to select modified cells and also provide a mechanism to deplete therapeutic cells in case of an adverse event. Some of these strategies have been combined with each other, with limited success.

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List of Abbreviations

^{3}H	Hydrogen^{3} labelled thymidine
41BBL	41BB ligand
^{51}Cr	Chromium^{51} labelled sodium chromate
Ab	Antibody
ACT	Adoptive cell therapy
ADCC	Antibody dependent cellular cytotoxicity
aFR	α–folate receptor
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
aAPC	Artificial antigen presenting cell
APC	Antigen presenting cell
ATCC	American Type Culture Collection
ATF	Activating-transcription factor
AZT-MP	3’-azido-3’-deoxythymidine monophosphate
AZT-TP	3’-azido-3’-deoxythymidine triphosphate
β2m	Beta-2-microglobulin
BFP	Blue fluorescent protein
BSA	Bovine serum albumin
CAIX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
Cas	CRISPR associated systems
CD	Cluster of differentiation marker
CDC	Complement mediated cytotoxicity
cDNA	Complementary deoxyribonucleic acid
CLL	Chronic lymphocytic leukaemia

CMV	Cytomegalovirus
CN	Calcineurin
COPI	Coat protein 1
CR	Complete remission
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR-RNA
CRS	Cytokine release syndrome
CsA	Cyclosporin A
CT	Cancer testis
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte associated antigen
Cy7	Cyanine-7
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco’s PBS
dPTCRA	Truncated pre-T cell receptor alpha
DSB	Double strand break
EBV	Epstein-Barr virus
ECACC	European collection of cell cultures
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
FACS	Fluorescence activated cell sorting

FBP	Folate-binding protein
FBS	Foetal bovine serum
FCS	Foetal calf serum
FDA	US food and drug administration
FHL	Familial haemophagocytic lymphohistocytosis
FITC	Fluorescein isothiocyanate
Flk	Foetal liver kinase
FK506	Tacrolimus
FKBP	FK506 binding protein
FMDV	Foot and mouth disease virus
Fv	Variable fragment
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
GMP	Good manufacturing practice
gp100	Glycoprotein 100
gRNA	Guide RNA
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
GvT	Graft versus tumour
HEK	Human endothelial kidney
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HMCV	Human cytomegalovirus
HR	Homologous recombination

HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplant
HSV	Herpes simplex virus
HSV-TK	Herpes simplex virus thymidine kinase
iCasp9	Inducible caspase 9
ICP47	Infected cell protein 47
IDT	Integrated DNA technologies
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin-like transcript
IMDM	Iscove's Modified Dulbecco's Media
IMGT	Immunogenetics database
IRE	Inositol-requiring enzyme
IRES	Internal ribosomal entry site
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
kDa	Kilodalton
KIR	Killer-cell immunoglobulin-like receptor
LB	Luria-Bertani broth
LMO2	LIM domain only 2
LMP	Latent membrane protein
LTR	Long terminal repeat
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
MART	Melanoma antigen recognized by T cells
MAS	Macrophage activation syndrome

MFI	Mean fluorescence index
mHag	Minor histocompatibility antigen
MHC	Major histocompatibility complex
mRNA	Messenger RNA
miRNA	Micro ribonucleic acid
MLR	Mixed lymphocyte reaction
MLV	Murine leukaemia virus
MM	Multiple myeloma
MN	Meganuclease
mRNA	Messenger ribonucleic acid
mTMPK	Mutated human thymidilate kinase
MTX	Methotrexate
NCI	National Cancer Institute
NEB	New England Biolabs
NHEJ	Non-homologous end joining
NHL	Non-Hodgkin’s lymphoma
NK	Natural killer (cells)
NLS	Nuclear localisation signal
ns	Not significant
NT	Non-transduced
nTCR	Native T cell receptor
ORR	Overall response rate
OSCC	Orapharyngeal squamous cell carcinoma
PBL	Peripheral blood T lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PD1	Programmed death-1
PDL1	Programmed death ligand-1
PE	Phycoerythrin
PEA	Pseudomonas exotoxin A
PEI	Polyethylenimine
PerCP	Peridinin chlorophyll
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PFA	Paraformaldehyde
PFS	Progression free survival
PHA	Phytohaemagglutinin
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PR	Partial remission
PTLD	Post-transplantation lymphoproliferative disease
RISC	RNA-induced silencing complex
RIT	Recombinant immunotoxin
RN	Retronectin
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RVD	Repeat variable di-residue
SAE	Serious adverse event
SB	Sleeping beauty
scFv	Single chain variable fragment
SCCHN	Squamous cell carcinomas of the head and neck
SCID	Severe combined immunodeficiency
SCT	Stem cell transplantation
SHLH	Secondary haemophagocytic lymphohistocytosis

SIN	Self-inactivating
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
TAA	Tumour-associated antigen
TAG	Tumor-associated glycoprotein
TALEN	Transcription activator-like effector nuclease
TAP	Transporter associated with antigen processing
TaV	Thosea asigna virus
TB	Terrific broth
TBE	Tris/Borate/EDTA buffer
TBI	Total body irradiation
TC	Tissue culture
TCR	T cell receptor
TGF	Transforming growth factor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	Tumour-infiltrating lymphocyte
TLS	Tumour lysis syndrome
TMD	Transmembrane domain
TNBC	Triple-negative breast cancer
TNF	Tumour necrosis factor
TRAC	T cell receptor alpha chain
tracrRNA	Trans-activating CRISPR RNA
TRBC	T cell receptor beta chain
TSS	Toxic shock syndrome
UPR	Unfolded protein response
US11	Unique short glycoprotein 11
UV	Ultraviolet

VEGF	Vascular endothelial growth factor
VH	Variable heavy chain
VL	Variable light chain
WBC	White blood cell
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-SCID	X-linked severe combined immunodeficiency
ZFN	Zinc finger nuclease

Chapter One:

Introduction

1.1 Cancer

Cancer is one of the most common causes of death globally, with one in four deaths in the US being due to cancer. In 2005, there were 7.6million deaths as a result of cancer, projected to rise to 11.4million by 2030 (Gavhane et al. 2011). In the US, in 2014 alone, it is estimated that there will be over 1.6million new cases, and over half a million deaths from the top ten types of cancer. The lifetime probability of being diagnosed with an invasive cancer is 44% for men and 38% for women (Siegel et al. 2014). As treatments for other diseases improve and more people survive, cancer becomes a more prevalent disease and cause of death.

Research and treatment of cancer stretches back for a couple hundred years, with Ephraim McDowell recorded as removing an ovarian tumour by surgery in 1809. Surgery was the main form of cancer treatment for almost a century until the era of radiation treatment that began in 1895 with the work of Roentgen and Pierre and Marie Curie. The cellular origin of cancer was discovered by Virchow in 1863. Chromosomal mutations in cancer were discovered by Boveri in 1914 and the National Cancer Institute (NCI) was set up in 1937. By the 1950s, only a third of cancers could be cured by the use of surgery, radiation or a combination of the two. Chemotherapy became available in the mid-1970s and now forms part of a three-pronged approach to tackling cancer. Despite the vast amount of research into cancer, its causes, symptoms and treatment, the relative survival rate only reached 68% in 2005, and some cancers are still as lethal as they were nearly 50years ago (DeVita & Rosenberg 2012). As with radiation and surgery though, chemotherapy is reaching the point where improvements in survival are difficult to achieve, and other avenues of treatment are being discovered. The most recent development is the use of monoclonal antibodies as targeted therapies, specifically targeting cancer-associated antigens. These have been shown to be highly effective when cancers express molecules differentially to healthy cells.

1.1.1 Solid Cancers

Solid tumours are abnormal masses of tissue that can be benign or malignant. Examples are sarcoma, arising from bone or muscle tissue, lymphoma, originating from lymphoid tissue, spleen or thymus and carcinoma, deriving from epithelial or glandular cells. As stated earlier, the main treatments for these types of cancers are surgery, radiation, chemotherapy and monoclonal antibodies. The main difference between solid cancers and healthy tissue is the vasculature of the tumour, with tumour vascular environments often containing distended capillaries with slow blood flow and leaky walls, leading to hypoxic conditions (Brown & Giaccia 1998). Discussed below are the obstacles to treatment of solid tumours and strategies that have been employed to overcome them.

1.1.2 Resistance to targeted small molecules

Chemotherapy has been the main method of treatment for over 50years, and has been shown to be effective for some cancers. The drawback is the high levels of toxicity associated with this treatment. Drugs used in chemotherapy often have debilitating side-effects, such as nausea, hair loss and tiredness (Love et al. 1989). When targeting the tumour cells, small molecules are not usually completely effective. As a result, there are tumour cells that are resistant to the small molecule, and therefore, once treatment is stopped, these cells recommence proliferating and cause a relapse in the patient. The same small molecule is therefore ineffective for a second treatment as the cells are all resistant to it. One example of standard chemotherapeutic treatment is that used for diffuse large B cell lymphoma. This treatment is comprised of cyclophosphamide, doxorubicin, vincristine and prednisone (collectively known as the CHOP regimen). Due to tumour resistance, this has a low complete response rate of 40-50% and three-year survival rates of 35-40% in elderly patients (Coiffier et al. 2002). Trying to add further cytotoxic drugs has not proved successful, as they require the doses of cyclophosphamide and

doxorubicin to be reduced, as well as having a higher level of associated toxicity (Fisher et al. 1993). As most patients diagnosed with diffuse large B cell lymphoma are elderly, any increase in drug toxicity is not well tolerated, and it appears that this therapy, used alone, is close to reaching the limit of its effectiveness.

Another obstacle to the use of small targeted molecules is the tumour microenvironment, which is often poorly vascularised and hypoxic. There are usually two types of blood vessels in the tumour – those around which the tumour formed initially, and those that have been generated as a result of neovascularisation caused by the tumour cells. As such, treatment of patients with solid tumours has had most success with antibodies that target antigens such as VEGF and EGFR (Scott et al. 2012). VEGF in particular has stood out as being promising as a target for anti-angiogenic treatment in tumours, either on its own, or in combination with molecules such as foetal liver kinase (Flk)-1 (W. S. Lee et al. 2015).

As well as the hypoxic environment, the poorly organised vasculature causes an increase in interstitial fluid pressure and an absence of fully functional lymphatics. The extracellular matrix (ECM) is also composed in such a way as to slow down movement of molecules within the tumour. This causes a reduction in the extracellular pH, again inhibiting efficacy of small molecule therapy. All of these factors combined together result in a limitation of small molecule distribution to tumour cells and often limit treatment to cells that are on the exterior of the tumour, or the cells closest to the vessels perfusing the tumour environment (Minchinton & Tannock 2006). Clearly solid tumours are difficult to treat and there are multiple obstacles standing in the way of an effective treatment, and whilst small molecules can be effective in certain cancer subtypes, they are very much limited as a general cancer treatment.

1.2 Immunotherapy

A fairly recent development in tumour therapy has been the use of immunotherapy. This is designed to manipulate the patient's own immune system and to augment the response elicited against tumour cells. Most commonly, autologous T cells are removed from a patient, and can be genetically modified to target cancer cells, or cancer-specific T cells can be expanded *ex vivo* before being reinfused to the patient. This has been shown to be successful in certain cancers, including melanoma, but requires immunogenic tumours in order to have tumour-reactive T cells to expand initially (Rosenberg et al. 2004). Other studies have shown that genetic manipulation by the introduction of native TCR or CAR transgenes can be successful at achieving tumour regression (Abad et al. 2008; Pule et al. 2008). Other cell types have been used in immunotherapy (**Table 1**), but T cells are the most studied and are effective at both cell killing and development of memory. Outlined below are some of the methods that have been used to exploit this recent avenue of cancer treatment.

Table 1 *– Cell types that have been used in immunotherapy*

Cell Type	Advantages of cell type	Reference
B Cells	Antibody factories, able to produce large quantities of fully human antibodies against target.	Moutai et al. 2014
Dendritic Cells	Integral cells in modulating immune response. Most efficient cell type at processing and presenting antigens.	Palucka & Banchereau 2013 Schuler et al. 2003
NK cells	Recognise multiple ligands that can induce a cytotoxic response. Short lifespan, do not require suicide mechanism.	Ames & Murphy 2014 Jewett et al. 2012
T cells	Potent killing mechanisms when activated by specific antigens. Able to develop memory and interact with other immune cell types.	Kalos & June 2013 Han et al. 2013

1.2.1 Check point inhibition

The immune system is a surveillance system that constantly 'scans' and monitors the homeostasis of the body. Within this system, immune cells are either activated by foreign or 'abnormal' molecules, or they are inhibited by recognition of checkpoint molecules. By removing or inhibiting some of these checkpoint molecules, the immune system can be activated so that it does respond to tumour cells that might otherwise evade recognition.

Two molecules that are involved as T cell checkpoint molecules are cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed death protein 1 (PD1). CTLA4 is exclusively expressed on T cells and interacts with the CD80 and CD86 on antigen presenting cells (APCs), competing with the activatory CD28 molecule. CTLA4 dampens the T cell activation signal as well as providing inhibitory signals to the T cell (Linsley et al. 1994; Riley et al. 2002; Sharma et al. 2011). The importance of CTLA4 as an inhibitory molecule are clearly demonstrated in CTLA4 knockout mice, which display lethal systemic immune hyperactivation (Waterhouse et al. 1995). From this, it was discovered that partial blockade of CTLA4 was able to achieve anti-tumour responses when anti-CTLA4 antibodies were used in mice (Leach et al. 1996). This led to the development of ipilimumab, a monoclonal antibody, which showed a 3.5 month survival benefit for patients with advanced melanoma compared to standard treatment without ipilimumab (Hodi et al. 2010).

PD1 is involved in limiting the activity of T cells in peripheral tissues during inflammatory responses and in autoimmunity (Ishida et al. 1992; Keir et al. 2006; Nishimura et al. 2001). PD1 expression is stimulated when T cells are activated, and inhibits kinases that are involved in T cell activation by the SHP2 phosphatase (Freeman et al. 2000). This is predicted to alter the duration of synapse formation, potentially reducing the overall activation signal (Fife et al. 2009). In contrast to CTLA4, PD1 is more widely expressed on B cells and NK cells, which also limits their lytic capabilities. Therefore, blockade of PD1 would likely affect B cell and NK cell activation also (Terme et al. 2011; Pardoll 2012). When testing anti-PD1 antibodies in Phase I clinical trials, initial results are

promising, with a mixture of mixed, partial and complete responses (Brahmer et al. 2010). There are other checkpoints that are yet to be, or currently undergoing, investigation. To broaden targets for treatment, it is also possible to target both the ligand-receptor pair of molecules. This has been demonstrated with PD1 and its ligand programmed death ligand (PDL)-1, where both receptor and ligand antibodies have been developed and tested in separate clinical trials with some clinical responses shown (Brahmer & Pardoll 2013). In recent months, several clinical trials have published data for anti PD1 therapies, including nivolumab, pembrolizumab and pidilizumab. These have all shown positive data, where overall response rates (ORRs) and/or overall survival (OS) rates have increased when treating patients with metastatic melanoma or advanced melanoma (Sznol et al. 2013; Ribas et al. 2013; Atkins et al. 2014). Both nivolumab and pembrolizumab were granted FDA approval in the last quarter of 2014 (Homet Moreno et al. 2015).

1.2.2 Tumour infiltrating lymphocytes

Tumour infiltrating lymphocytes (TILs) are white blood cells found in tumours. They comprise of various subtypes of lymphocytes, but are known to contain tumour-reactive T cells (Holmes 1985; Dudley et al. 2008). It is possible to extract these TILs and to expand them *ex vivo* before giving them back to the patient. The protocol for this requires isolation of TILs from patient biopsies followed by progressive selection of tumour-reactive cells using high levels of IL-2 and other cell culture techniques (June 2007). Success has been shown in the use of TILs to treat metastatic melanoma, with partial and mixed responses obtained. Thirteen patients were pre-conditioned with the immunosuppressive drug combination of cyclophosphamide and fludarabine, followed by treatment with TILs. Six patients had partial responses; four had mixed responses (Dudley et al. 2002). Importantly, prolonged engraftment was demonstrated compared to patients who did not receive pre-conditioning therapy, demonstrating the efficacy of combination treatments contrasted to single strategy therapies. A recent study has also shown the efficacy of TILs

in generating tumour responses against cervical cancer, where T cell populations were chosen based on oncoprotein reactivity, T cell growth rate and $CD8^+$ T cell number. Three of seven patients demonstrated tumour responses; two demonstrated complete and ongoing tumour regression at three and nine months after treatment (Hinrichs et al. 2013).

There are drawbacks to this therapy though. Less than 50% of biopsies yield usable T cell populations and the T cell expansion protocol is both cytokine-intensive and takes up to six weeks (Pedrazzoli et al. 2012; Dudley et al. 2003). In the study by Hinrichs et al. (2013), adverse events were seen due to the toxicities of the preparatory immunosuppressive treatment and the IL-2 administered alongside the TILs. Administration of TILs, therefore, seems to carry risks of toxicity as wells as taking a long period of time to generate them in the first place.

More recently, instead of a therapy, TILs have been used as an indicator of therapeutic success of other treatments. The presence of TILs indicates an immune system that is primed to react to the tumour and is easier to trigger using therapeutic regimens compared to an immune system that contains low or no TILs. It has been suggested that TILs could be used as part of a prognostic analysis in squamous cell carcinomas of the head and neck (SCCHN), oropharyngeal squamous cell carcinoma (OSCC), triple negative breast cancer (TNBC) and predictive for the effects of trastuzumab in early breast cancer (Jones 2014; Loi et al. 2014; Adams et al. 2014).

1.2.3 T cell receptor transfer

TCR transfer was developed to overcome some of the shortcomings of TILs. By having a transgenic TCR ready to introduce to donor or autologous T cells, any T cells can be extracted and modified quickly, with protocols as short as two to three days possible depending on the method of gene transfer used. Use of transgenic TCRs also allows for the use of TCRs that are not normally

found in the endogenous TCR repertoire, providing a wider range of targets than possible with therapies such as TILs (Schumacher 2002).

The first co-transfer of α and β chains from a TCR to confer specificity against an antigen by polyclonal T cells was performed by Dembić et al. (1986). The main restriction on the use of TCRs is on the HLA typing. Since HLA-A2 is the most common HLA type in the Caucasian population and HLA-A24 is the most common HLA type in the Japanese population, TCRs restricted to these HLA molecules have been widely investigated (Casucci et al. 2012). Abad et al. (2008) have shown, in developing a murine melanoma model, that transgenic expression of a TCR specific for a cancer antigen can also be used as a method of therapy. Since then, a study has shown that autologous lymphocytes that express a TCR against a tumour-associated antigen (TAA) are able to mediate tumour regression (Rosenberg et al. 2008). TCRs have also been generated against the WT1 antigen, the minor histocompatibility antigens HA-1 and HA-2, melanoma antigen recognized by T cells (MART)-1, glycoprotein (gp)-100, NY-ESO-1 and p53 (Xue et al. 2010; Mommaas et al. 2005; Morgan et al. 2006).

Another limitation of TCR transfer is the risk of the α and β chains cross-pairing with endogenous TCR chains, leading to exogenous-endogenous TCR chain pairing. This could result in a lower avidity for the target of choice, as well as having the potential to create auto-reactive TCRs, which has been demonstrated in a mouse model with lethal cytokine-driven toxicity (Bendle et al. 2010). This risk can be minimised by strategies such as the use of codon optimisation, small interfering ribonucleic acid (siRNA) to reduce endogenous TCR expression or introducing a cysteine pair to the transgenic TCR (Kuball et al. 2007; Okamoto et al. 2009; Schumacher 2002).

1.2.4 Chimeric antigen receptor transfer

CARs, in comparison to chemotherapy, radiotherapy and mAbs, are a relatively recent development. They comprise the heavy (V_H) and light (V_L)

variable fragments from an antibody, joined by a short linker, to form a single chain variable fragment (scFv). This is attached to a spacer region to protrude the molecule from the cell surface. This is attached to a transmembrane domain (TMD), which is then subsequently attached to one or more intracellular activation domains. These may be immunoreceptor tyrosine-based inhibition motifs (ITIMs) or immunoreceptor tyrosine-based activation motif (ITAMs) depending on what intracellular signalling pathway is being activated or inhibited. First generation CARs contained one intracellular signalling domain, with second generation CARs containing one additional costimulatory domain, and third generation CARs containing two costimulatory domains. First generation CARs showed limited efficacy, but lacked *in vivo* persistence and activity. Second and third generation CARs have been created to resolve these issues, and many different combinations of intracellular costimulatory domains are in clinical trials, which will help determine the most effective combination (Dotti et al. 2009). Most commonly used are the CD3ζ, CD28, OX40 and 41BB intracellular signalling domains. These constructs are introduced into T cells through the use of viral carriers, and then are used to re-direct the antigen specificity of the T cell (Willemsen et al. 2004).

CARs are able to target cell surface antigens without needing major histocompatibility complex (MHC) recognition (Sadelain et al. 2009). As such, they are able to modify most subsets of T cells with ease, leading to increased persistence and improved development of memory (Curran et al. 2012). In addition, they are not restricted to recognition of protein-derived peptides, they can recognise every cell surface molecule on a target cell, including nonprotein structures such as gangliosides and carbohydrate antigens (Mezzanzanica et al. 1998).

One clinical trial in 2008 was able to modify Epstein-Barr virus (EBV)-specific cytotoxic lymphocytes (CTLs) with an anti-GD2 CAR. The stimulation provided by the EBV-CTLs, along with the anti-GD2 CAR, resulted in enhanced activity and persistence of the transferred cells, and tumour regression was shown in half of the patients treated (Pule et al. 2008). A more recent clinical trial has shown that anti-CD19 CARs, generated using a 10day protocol, have been

successful at mediating five complete remissions and six partial remissions out of fourteen patients receiving pre-conditioning therapy followed by CAR infusion, without the addition of exogenous IL-2 (Kochenderfer et al. 2013). A recent clinical trial into the treatment of ALL has shown high levels of efficacy as a result of autologous aCD19-CAR-modified T cells, with complete remission (CR) demonstrated in 90% of patients. Durable remissions were seen for up to 24months, and treatment showed efficacy even amongst patients who had undergone failed stem-cell transplantation (Maude, Frey, et al. 2014). This clearly shows the vast potential of CAR-mediated therapy and its benefits of high affinity molecules, lack of HLA restriction, reduced toxicity, shortened cell manipulation time, and functional efficacy *in vivo*.

1.3 Other cancer therapies

Current treatments for B cell malignancies include chemotherapy, radiotherapy, monoclonal antibodies and CAR therapy. Combination therapy of two or more of these strategies often proves to increase the efficacy of the treatment, but care has to be taken to ensure that the effects of toxicity do not outweigh the benefits achieved through combination therapy. The combination of trastuzumab, paclitaxel and carboplatin treatment for women with HER-2–overexpressing metastatic breast cancer (MBC) was evaluated in a phase III clinical trial and demonstrated to increase ORR and PFS in patients who were treated in this trial (Robert et al. 2006). So while individual therapies on their own have limitations, the combination of the therapies described below still has potential to ensure that cancerous cells can be targeted from as many directions as possible. There are multiple molecules that have been selected for targeted therapy of B cell malignancies, including CD20, CD23 and CD52, mainly using monoclonal antibodies (mAbs) (Schnaiter & Stilgenbauer 2010). CD19 and CD20 are expressed on B cell lineage cells, CD23 on follicular B cells and CD52 on mature lymphocytes. Currently, the main antigen targeted by various therapies is CD19.

1.3.1 Radiotherapy

Another form of cancer therapy is to use radiotherapy to destroy cancerous cells by inducing deoxyribonucleic acid (DNA) damage. The main drawback with this treatment is that there is no discrimination between healthy cells and cancerous cells if ionizing radiation is used. Chronic lymphocytic leukaemia (CLL) is very sensitive to radiotherapy in the early stages of disease (I-II). Most patients, however, are diagnosed with stage III-IV disease, which is much more resistant (Jóhannsson et al. 2002). Total body irradiation (TBI) has been a common treatment, but has not been shown to be any more effective than chemotherapy. Radiotherapy has commonly been used to treat breast cancer, and has been shown to be effective, but has increased risks of cardiac and pulmonary toxicity, amongst other side effects (Brown et al. 2015). There are also limitations on the size of tumours that can be treated with radiotherapy. In an investigation into the incidence of adverse radiation effects after radiation therapy for brain metastases, risk increased rapidly with an increased size of lesion and tumour, demonstrating another limitation of this type of treatment (Sneed et al. 2015).

1.3.2 Monoclonal antibodies

Monoclonal antibodies (mAbs) are commonly derived from antibody-producing hybridoma cells. A study in 1976 demonstrated that antibodies could be produced against any antigen and this was followed by the ability to fuse antibody-producing cells from mice with myeloma cells to create hybridomas (Klinman et al. 1977; Köhler & Milstein 1975). Over time, more mAbs have been discovered and developed, and if derived from a non-humanised platform, they can now be modified and humanised through genetic engineering to be 95% human (O'Mahony & Bishop 2006). mAbs can also be isolated from donors who have been exposed to high levels of antigens, such as nursery workers, who have been shown to have high titres of viral-specific B cells as a result of exposure (Chan-Hui 2013). Animal models can be

generated that produce fully human antibodies, using technology developed by companies such as Kymab Ltd., who have mice with a fully human antibody repertoire (Lee et al. 2014). These mAbs can be conjugated or unconjugated to another molecule. Unconjugated antibodies carry out their effects through complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC), by blocking receptor-ligand interactions or by inducing cellular death. Conjugated antibodies are used as carriers for various molecules, including radioactive isotopes, as mentioned in the previous section, in order to deliver them to the antigen of choice. It is also possible to conjugate toxins, which can then be internalised and carry out their effects.

In the treatment of B cell malignancies, multiple mAbs have been used, including ibritumomab, rituximab and alemtuzumab. In order to choose the best antigen to target, a mAb needs to meet three main criteria. Firstly, it needs to be almost exclusively expressed on the cell of choice, to reduce or prevent the risk of on target, off-site toxicity. Ideally, the antigen also needs to be a molecule that has constant surface expression levels to ensure effective depletion of the target cells. Finally, the antigen should not have soluble forms otherwise the mAb will have further off-site activity and reduce the effectiveness of treatment.

Rituximab targets the CD20 antigen. It is a combination of the murine variable regions from the ibritumomab, with the human immunoglobulin (Ig)-G1 constant regions (Golay et al. 2000). It works through CDC, ADCC, inhibiting proliferation, inducing apoptosis and sensitising cells to chemotherapeutic agents (Demidem et al. 1997). In 1998, it was the first mAb approved by the FDA for use in cancer treatment. A study by McLaughlin et al. (1998) showed an overall response rate (ORR) of 48%, with 42% partial remissions (PR) and 6% complete remissions (CR). A small percentage of patients remained in remission that was unmaintained for more than five years.

Alemtuzumab is a humanised IgG1 mAb that targets CD52, which is expressed on mature B cells and T cells as well as spermatozoa. Most B cell neoplasms express the CD52 antigen, including B cell CLL. Alemtuzumab mediates its effects by ADCC and CDC. In comparison to rituximab,

alemtuzumab does not require complement activation in order to deplete CD52-expressing cells (Golay et al. 2002). As a result, it is not as effective as rituximab if using cell lysis as the outcome. It has been combined with rituximab in clinical trials, and has been shown to be useful in the control of graft-versus-host-disease (GvHD) after allogeneic haematopoietic stem cell transplantation (HSCT) (Kottaridis et al. 2000).

1.3.3 Recombinant immunotoxins

Recombinant immunotoxins (RITs) are fusion proteins, where the cell-binding protein of a bacterial toxin has been replaced by the variable fragment (Fv) of a mAb. The heavy and light chains from an antibody are linked together by a flexible peptide linker or they are stabilised by a disulphide-bond. This is then fused to the immunotoxin of choice and then cloned into an expression vector. Proteins are expressed and prepared in inclusion bodies, which are then used to treat target cells. This results in being able to use the toxicity of a bacterial toxin and the targeting of an antibody to target cancer cells. The toxins used can be very potent and they can kill cells that are otherwise resistant to chemotherapy (Pastan & Ho 2010). One toxin, Pseudomonas exotoxin A (PEA), has been in clinical trials with CD22 and CD25 targeting Fvs, as well as mesothelin-expressing solid tumours (Kreitman et al. 2001; Kreitman et al. 2000; Hassan et al. 2007).

1.3.4 Radioimmunotherapy

Combining radiotherapy with mAbs has proven to give higher response rates than using them individually. To try and reduce off-target effects, recent treatments have attached a radioactive isotope to an antibody targeted to a cancer antigen to develop radioimmunotherapy as a combination treatment (Witzig et al. 2002). This treatment uses the antibody as a carrier molecule to distribute the radioactive isotope to the cancerous cells. It requires an antibody

that targets an antigen that is specific to the cancer cells. As a result, much of the ionising radiation will be received by the cancerous cells, but there will still be some damage caused to healthy cells as the antibody travels through the body to the target site.

Ibritumomab is a murine mAb which targets the CD20 antigen, which is expressed by all B cell lineage cells, but not on early progenitor lymphocytes (O'Mahony & Bishop 2006). CD20 is stably expressed on the cell surface and is not prevalent as a soluble molecule, making it a good candidate for B cell targeting. Ibritumomab tiuxetan was the first radiolabelled mAb to be approved by the US Food and Drug Administration (FDA) in 2002 for therapeutic use, using yittrium 90 (^{90}Y) as the radioisotope. In a study by Knox et al. (1996), patients were pre-treated with unconjugated ibritumomab before being treated with up to 50mCi of labelled mAb. This resulted in minimal haematological toxicity and promising clinical responses. Progression-free survival (PFS) was 72% for 3-29+ months and 78% PFS for doses of up to 40mCi. When compared to rituximab, the ORR was higher (80% compared to 56% for rituximab), and the CRs were higher (30% compared to 16% for rituximab) (Witzig et al. 2002).

1.4 Creation of a universal T cell

Although there has been much recent progress in the treatment of cancer, patients can go on to develop a chemotherapy-resistant disease. Older patients suffering from acute lymphocytic leukaemia (ALL) also have a low chance of survival, although this is not the case for children treated with T cells expressing a CD19 CAR (D. W. Lee et al. 2015). Single therapy is reaching the limit of its effectiveness and combination therapy has been shown to be more effective in the treatment of malignancies and in providing sustained protection against relapse. Adoptive immunotherapy is an attractive avenue to explore in order to develop these treatments further in malignancies such as CLL and ALL.

Adoptive immunotherapy with genetically engineered T-cells is showing much promise. Adoptive immunotherapy is the use of immune cells to elicit an immune response against a target; in this case, cancerous cells. Theoretically, creating a cell-based product is the least toxic treatment that can be developed as its aim is to closely mimic the mechanisms of action of the immune system. In a healthy individual, T cells move through the body scanning for MHC-peptide complexes that activate their TCRs. In the case of infection, peptides are recognised as being foreign, allowing an immune response to be activated. In the case of cancer, the affected cells predominantly express self-peptides or down-regulate MHC expression, and are therefore unlikely to be recognised by T cells or APCs. There are some tumour-reactive T cells that are most likely activated by tumour-associated antigens (TAAs) presented by APCs, and adoptive immunotherapy aims to either augment this immune response or provide cells that can mimic this (Restifo et al. 2012).

1.4.1 Steps required in creating a universal T cell

At present, a bespoke therapeutic T-cell product has to be made for each patient either from autologous cells, or in some protocols which involve HSCT, from an allogeneic donor. Using autologous cells has problems: patients may not have healthy, readily-available T cells to isolate and transduce, especially if they are immuno-compromised, usually as a result of disease or lymphodepletion. This includes the additional risk of obtaining and engineering malignant cells, potentially increasing the persistence of the related malignancy in the patient. This method also requires a different cell product to be made for every patient (Abken et al. 1998). It can take several weeks for T cells extraction, *in vitro* manipulation and clinical preparation of the cells before they can be administered back to the patient. From an allogeneic donor, cells are more likely to be disease-free, but have the obstacle of HLA-restriction, the risk of GvHD, along with the possible need for immunosuppression (Vonderheide & June 2014). Cell numbers required for these therapies vary

depending on cell source and efficacy, but typically range from 10^6-10^8/kg (Drobyski et al. 1993; Grupp et al. 2013; Doubrovina et al. 2012).

Adoptive cell therapy (ACT) has already shown promise in clinical situations for infections and malignancies. Graft versus leukaemia (GvL) has been shown in HSCT (Randolph et al. 2004), tumour-infiltrating lymphocytes have been used against metastatic melanoma (Rosenberg et al. 1988), and a clinical trial into neuroblastoma has shown that ACT can reconstitute EBV immunity following HSCT (Dudley & Rosenberg 2003). Despite this, it is relatively difficult to generate an effective, homogenous therapeutic T cell product using traditional methods of selection and expansion. The problem with using these methods is that cell viability drops off very rapidly once they have been removed from the *in vivo* context. Having to carry out multiple rounds of selection on this product then means that the cell product is non-homogenous and the quantity of healthy, re-directed cells is minimal. Gene therapy opens several other possibilities for incorporation of various elements, such as resistance genes, homing genes and suicide genes to allow for sorting, engrafting and selective deletion of therapeutic cell products (Grignani et al. 1998). It also allows the use of integrating and non-integrating vectors to re-direct cell products, which are relatively straight-forward to generate and introduce into cells.

Gene therapy is not without its own drawbacks. By transferring T cells in an allogeneic setting, there is the risk of GvHD, caused by the endogenous TCR expressed on the therapeutic cells, along with the rejection of the therapeutic cells via the recognition of mis-matched HLA (major and minor), by the recipient's immune system. CARs have the risk of being immunogenic, with anti-CAR antibody responses observed, ranging in severity (Sadelain et al. 2009). The CAR may also cause some toxic off-target effects, so target selection has to be robust. The method of genetic integration used also comes with some associated problems. Retroviral and lentiviral vectors are the two most common integrating methods used in gene therapy. They do carry with them the danger of insertional mutagenesis, which can potentially cause the initiation of leukaemia, although this risk is minimal (Uren et al. 2005). The advantage of these vectors is that they have a relatively large packaging

capacity, meaning that it is possible to incorporate multiple elements into one vector. In recent years, the development of third generation CARs, along with research into suicide genes and the discovery of new potential cancer targets, has allowed the potential of ACT to grow. Combined with improvements in methods of gene delivery, ACT has now become a realistic alternative to drug development.

1.4.2 Obstacles to creating a universal T cell

In order to create a universal T cell, several obstacles need to be overcome. These include GvHD caused by allogeneic T cells, NK-mediated null cell killing of HLA-null T cells, host immune response to allogeneic cells, re-direction of the T cell towards a TAA, selection of modified cells to create a homogenous cell product and a mechanism to deplete therapeutic cells in case of severe adverse events, including GvHD. These all involve the assembly and testing of constructs aimed at resolving these issues, which is the aim of this project.

1.4.3 Graft-versus-Host Disease

Since the 1950s, stem cell transplantation (SCT) has been investigated as a treatment for patients following radiotherapy or chemotherapy. Before this, it was known that while aiming to achieve haematopoietic recovery, transplantation of splenocytes from noncongenic donor strains caused severe illness in mice and guinea pigs, displaying weight loss, diarrhoea and a hunched posture (Lorenz et al. 1951). Since, it has become clear that this is due to an immune-mediated activity (Wolf et al. 2012). Pre-conditioning before SCT is used to treat malignancy and to suppress the immune system of the recipient to prevent graft rejection. Allogeneic SCT involves using cells from matched or mis-matched donors and provide an additional graft-versus-tumour (GvT) effect in some cases. In GvHD, immunocompetent donor cells recognise host cells as foreign. Therefore, the greater the HLA mis-match

between donor and recipient, the greater risk there is of GvHD (Hymes et al. 2012).

GvHD can be classified as being either acute or chronic. Acute GvHD has been traditionally defined by the onset of symptoms within the first 100days of a transplant, with chronic GvHD defined as showing symptoms more than 100days post-transplant. With the development of transplant protocols and varying conditioning regimens, there has been a need to revisit these definitions. Acute GvHD may present after 100days and in patients treated with donor lymphocyte infusions (DLIs), symptoms of acute and chronic GvHD can be present together (Filipovich et al. 2005). Complications in diagnosis arise from the need to eliminate other causes for symptoms, such as infections and drug toxicities.

Animal models have been used to gain a clearer understanding of the pathophysiology of acute GvHD. From these models, a three-stage process has been derived. Firstly, tissue damage, as a result of conditioning chemotherapy or radiotherapy, occurs. This leads to a release of inflammatory cytokines. Secondly, from the graft, mature lymphocytes enter into this environment, leading to their activation and proliferation upon encountering host and donor APCs. Thirdly, alloreactive T cells proliferate into cytotoxic T cells that cause tissue damage and further cytokine release (Ferrara et al. 2009).

The pathophysiology of chronic GvHD is much harder to understand due to a lack of suitable animal models that replicate the complex conditions. The current models are often based on autoimmune disease models, which involve isolated organ involvement as opposed to the multi-system effects of GvHD. It is known that GvHD has an abundance of detectable autoantibodies, including antinuclear, double-stranded DNA and smooth-muscle antibodies (Quaranta et al. 1999). This is a similar scenario to that found in classic autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Ippolito et al. 2011; Song & Kang 2010).

1.4.4 Host immune response

In the case of SCT, HSCT or solid organ transplant, host T cells recognise the foreign HLA peptides expressed on donor cells, causing an immune response. The large HLA locus encodes genes for Class I, II and III MHC, which are responsible for presenting intracellular antigens (Class I), extracellular antigens (Class II) and activation of the complement system (Class III) (Hewitt 2003; Neefjes et al. 2011; Carroll et al. 1984). Recognition of foreign HLA by the recipient would trigger an immune response and destruction of the therapeutic T cells, negating any potential therapeutic effect that they may have had.

There are two main mechanisms by which this occurs. Firstly, 'direct' allorecognition, where T cells are activated by intact HLA molecules on donor APCs. Secondly, there is 'indirect' allorecognition, where T cells recognise foreign peptides that have been presented on self APCs. This indirect recognition is the same mechanism used in response to a viral or bacterial infection. The mechanism of direct recognition is specific for allogeneic transplants. Semi-direct recognition is also believed to be involved, where intact donor MHC molecules are transferred from donor cells to recipient dendritic cells (Podestà et al. 2015; Bolton & Bradley 2015).

Direct allorecognition is most likely to occur early after transplantation. Host helper T cells get into contact with donor APCs. This process takes precedence over indirect recognition at this stage due to the large number of T cells that recognise unprocessed foreign MHC-peptide complexes (Douillard et al. 1999; Auchincloss Jr & Sultan 1996; Benichou & Thomson 2009). A study by Benichou et al. (1999) demonstrated that over 90% of the response by T cells was directed to intact MHC molecules, while T cells going by the indirect recognition pathway were below 10%.

Later on after the elimination of donor APCs (mainly dendritic cells), the balance shifts from direct to indirect allorecognition. The recipient APCs infiltrate the donor graft and process allo-antigens (Harris et al. 1999; Ali et al. 2013). Studies have demonstrated that peptides derived from MHC molecules

make up a substantial portion of the naturally processed antigens that are presented by APCs (Shoskes & Wood 1994; Breman et al. 2014). This was further demonstrated when MHC Class I knock-out mice were shown to be able to reject skin grafts from MHC Class II knock-out mice. The recipient mice lacked CD8+ cytotoxic T cells capable of direct MHC Class I recognition, but did have CD4+ helper T cells that were stimulated indirectly by antigen presented in the context of MHC Class II molecules on APCs (Auchincloss et al. 1993).

Since helper T cells mainly recognise antigen presented on MHC Class II molecules, and cytotoxic T cells recognise antigen presented on MHC Class I molecules, it has been postulated that if the mis-match between donor and recipient is mainly based on HLA Class I, then the cytotoxic T cell response will be stronger and the direct allorecognition pathway will be dominant; conversely, if the mis-match is mainly in the HLA Class II alleles, then the helper T cell response will be stronger and the indirect allorecognition will play a larger role in the allograft rejection (Slavcev 2001).

1.4.5 Natural Killer cell-mediated killing of HLA class I null cells

NK cells have a range of activating and inhibitory receptors, with relevant ITAM or ITIM domains, to enable a range of functions. Receptors with ITAMs include the killer-cell immunoglobulin-like receptor (KIR)-2/3DS family, CD16 and CD94. Receptors with ITIMs include the KIR2/3DL family, NKG2A and T cell immunoreceptor with Ig and ITIM domains (TIGIT). There are still further receptors with unknown ligands and functions (Bryceson et al. 2011). Originally thought to be large granular lymphocytes, NK cells are now known to be of a separate lineage (Vivier et al. 2008). They have cytotoxic capabilities through the use of perforin-dependent cell killing and they are also able to produce cytokines (Trinchieri 1989). They patrol the body scanning for activating antigens, or for self-MHC molecules that will prevent activation and inhibit release of lytic granules. If they interact with a cell that is not expressing a HLA Class I molecule (a ‘null’ cell), they will be activated. This seems to be

a fairly robust mechanism, especially as various cancers down-regulate HLA Class I, including acute myeloid leukaemia (AML), ALL and CLL, yet these tumours manage to avoid NK cell killing through the expression of a non-classical HLA molecule, HLA-G (Menier et al. 2008; Nückel et al. 2005; Mizuno et al. 2000; Poláková et al. 2003). There are also some healthy cells that do not HLA Class I molecules, but avoid NK cell killing, including trophoblasts, which express HLA-G (Hunt & Langat 2009; Schust et al. 1998). NK cells are also activated by upregulation of activating ligands, which interact with the NKG2D receptor. Cancerous cells are also able to suppress expression of these ligands also (Eisele et al. 2006; Mincheva-Nilsson & Baranov 2014).

There are two main subsets of NK cells, $CD56^{dim}$ $CD16^{+}$ and $CD56^{bright}$ $CD16^{+}$ NK cells. The $CD56^{dim}$ $CD16^{+}$ NK cells make up around 90% of the NK cells in peripheral blood and the spleen. These cells express perforin, are cytotoxic and produce interferon (IFN)-γ upon encountering tumour cells (Anfossi et al. 2006). The $CD56^{bright}$ $CD16^{+}$ NK cells lack perforin and are mainly located in lymph nodes and tonsils and primarily express cytokines, including IFNγ upon stimulation with IL-12, IL-15 and IL-18 (Ferlazzo & Münz 2004; Cooper et al. 2001).

NK cell recognition is a complex process that involves a combination of activating and inhibitory interactions. The integration of these interactions and the subsequent signals determine on whether the NK cell detaches or whether it responds (Lanier 2005). As explained above, this response can be to produce cytokines, or to cause polarisation of lytic granules to the immunological synapse, which are then released onto the membrane of the target cell. NK cells differ from naïve T cells in that they are prepared to respond with prestored cytokines, lytic granules and granzymes, all of which can be released within minutes (Stetson et al. 2003). NK cells have a vast array of receptors, of which only a few ligands are well characterised. As a result, the complete mechanisms of NK cell action are not fully known, including how NK cells interact with other cells of the immune system during immune responses, the detachment of NK cells to allow serial killing to progress, and how activation thresholds are determined (Lanier 2005; Bryceson et al. 2011).

1.4.6 Re-direction of T cells

In T cell immunotherapy, there are two main routes to obtain T cells that are specific for a TAA. Firstly, T cells can be extracted and exposed to the TAA. This will cause tumour-specific T cells to divide and multiply. The drawback to this is that it is time-consuming, costly and causes the T cells to age, reducing their efficacy when reinfused. Secondly, gene transfer techniques can be used to introduce native TCRs (nTCRs) or CARs. These molecules target a specific TAA and cause the modified T cell to activate upon interaction with their target. This technique is quicker and enables all modified T cells to be reinfused.

TCRs are heavily expressed on T cells, each having been selected during differentiation of immune cells to react to foreign peptides and to allow tolerance of self-peptides. The huge variability in specificity caused by random splicing of the variable regions, forming the basis of the immune system. This is obviously beneficial in terms of health, but when using adoptive transfer of allogeneic cells, it can be a complex barrier to negotiate. nTCRs are MHC-restricted molecules, and are therefore limited to recognising antigens in the context of HLA molecules, either expressed on the tumour cell surface or on APCs. In contrast, CARs are not MHC-restricted, and can respond to tumour cells that have often down-regulated their MHC expression. As a result, this technology has the capability to recognise any cell surface molecule, including glycolipids, carbohydrates or soluble ligands. CARs are unable to access intracellular antigens though, and they are limited to using characterised antibodies.

The first effective use of nTCRs being used to target a TAA was demonstrated when using complementary DNA (cDNA) for a TCR specific for the m9-27 peptide of MART1 to target melanomas. Human donor peripheral blood T lymphocytes (PBLs) were engineered with this cDNA, and $CD8^+$ T cells generated from HLA-$A2^+$ donors were shown to lyse HLA-$A2^+$ melanoma cells *in vitro* (Clay et al. 1999). As previously mentioned, this strategy needs to be in the context of MHC-peptide complexes, and therefore, if used in an

allogeneic context, patient blood-typing needs to be carried out to find a matched donor. Also, for effective stimulation of an immune response, both $CD4^+$ and $CD8^+$ TCRs would need to be introduced. There is also the risk of forming hybrid TCRs with one chain from the introduced TCR dimerising with one chain from the endogenous TCR, leading to unknown off-target activity and potential autoimmune disease (Sadelain et al. 2003).

CARs have been developed as an alternative to nTCRs. CARs combine antigen specificity and a signalling domain in one molecule, performing the task of the TCR and CD3 complex. A CAR is made up of a targeting molecule, such as a scFv, peptide, cytokine, toxin or other moiety, protruded from the cell surface by a spacer, which may be a molecule such as the CD8 stalk. A TMD then links the extracellular ectodomain to the intracellular signalling molecules, which include the CD28 co-stimulatory molecule, the CD3ζ signalling domain and other signalling moieties, depending on the engineer (Altvater et al. 2010). Ligand binding to the CAR initiates phosphorylation of the ITAMs, leading to activation of the intracellular signalling cascade and T cell activation. In vivo efficacy of CAR-modified cells has been shown for various cancer antigens, including tumour-associated glycoprotein (TAG)-72, folate-binding protein (FBP) and human epidermal growth factor receptor (HER)-2 (Sadelain et al. 2003). Human PBLs targeted to the CD19 antigen have also demonstrated elimination of Burkitt's lymphomas after adoptive transfer to severe combined immunodeficiency (SCID) mice (Brentjens et al. 2002). Along with a wider range of potential targets and the lack of MHC restriction, CARs are not at risk of forming hybrid TCRs. CARs do have limitations though, including being restricted to surface-bound molecules, a need to be characterised fully to determine the strength of activation after ligand binding, and their potential for immunogenicity (Sadelain et al. 2003; Hombach et al. 2001; Beecham et al. 2000).

1.4.7 Selection of genetically engineered cells

When introducing genetic modifications into cells, there will be some cells that are successfully modified and some that will remain unmodified. If this mix of cells is used in immunotherapy, the efficacy of the treatment will be reduced, and there is a risk of unknown side effects and toxicities. Therefore, cells that have been genetically modified need to be selected to create a homogenous cell product. Traditionally, this has been done by fluorescence activated cell sorting (FACS), based on marker gene expression such as green fluorescent protein (GFP) and blue fluorescent protein (BFP), or by using antibiotic selection to select cells based on their expression of a resistance gene.

Antibiotic, or drug, selection has been used to allow for selection of cells that express the desired vector; one which includes the relevant resistance gene. After integration of the vector into the cell, usually by viral transduction, cells are positively selected by being cultured in the relevant antibiotic or drug (Kawahara et al. 2003). The drawback of this method is if the conditions used in the selection are incorrect, then this can lead to the killing of the desired population or to the survival of the unmodified population (Brielmeier et al. 1998). Selection of cells in this way also leads to impaired growth of the selected population. A study by Kim et al. (1998) demonstrated that the growth rate of cells grown in methotrexate (MTX) was reduced compared to cells grown in antibiotic-free medium.

Selection of cells, based on fluorescent marker genes or antibodies, uses FACS to select cells. Cells can be selected based on size, fluorescence or on cell surface marker expression. Fluorescence is based on the expression of a fluorescent marker gene, such as BFP and GFP, or by using an antibody, conjugated to a fluorophore, bound to the surface of a cell. Cell surface marker expression was used to sort ex vivo expanded T regulatory cells, using multiple cell surface markers to sort a specific cell population (Trzonkowski et al. 2009). Cells are placed into a stream of fluid that restricts them to a flow that is one cell wide. The nozzle of the machine vibrates, causing the stream to break up into droplets that each contain a single cell. A laser interrogates each cell to determine fluorescence and a charge is applied to the cell. Electromagnets cause the droplet with the charged cell to be deflected into a reservoir, and cells are sorted (Herzenberg et al. 1976). This is a very robust

method, but the cells need to be in a single cell suspension for successful sorting to occur. This makes it more of a challenge to sort adherent cells or cells from solid tissue. Another drawback is the speed of cell sorting when using FACS machines. For clinical trials or experiments involving model organisms, large numbers of cells are usually required. This requires large numbers of cells which take a long time to be sorted, leading to questions over the quality of cells that have been out of optimal growth conditions for many hours. The sterility of the FACS machine also needs to be maintained between each cell sort to prevent cross-contamination (Cammareri et al. 2008).

A fairly recent innovation in cell sorting is magnetic cell sorting (MACS). To do this, antibodies attached to magnetic beads are used. Cells are incubated with the antibody-bead conjugate and then passed through a column attached to a magnet. Cells that have the antibody-bead conjugate attached are retained in the column, and unlabelled cells pass through. Once the column has been washed with buffer, the column is removed from the magnet and the labelled cells are eluted. The remaining magnetic beads are biodegradable and degrade in culture. This allows for quick, sterile sorting of cells based on the expression of a cell surface marker (Cammareri et al. 2008). Again, this method works optimally with cells in a single cell suspension, and the choice of selection marker needs to be careful, for example, selecting NK cells using anti-CD56 beads may activate the NK cells, or select NKT cells as well.

1.4.8 Mechanism for the depletion of therapeutic cells

Genetic engineering of T cells with TCRs or CARs has expanded the range of malignancies that can be treated using ACT. Extensive modification of T cells by viral gene transfer could have the potential to alter their activation status and *in vivo* survival. The enhanced capacity to engraft and to persist *in vivo* longer than previous treatments using peptides or chemotherapeutic agents has previously been demonstrated (Louis et al. 2011). GvHD and other toxicities have been demonstrated in previous studies involving genetically

engineered T cells and further toxicities have been suggested in animal models (Morgan et al. 2010; Brentjens et al. 2010; Bendle et al. 2010).

Cell-based therapy also carries the risk of over-activating the immune response of the recipient. As a result, there needs to be a mechanism by which therapeutic cells can be selectively removed from the patient to prevent the development of serious adverse events (SAEs). This can be done through the use of mAbs or drugs that specifically target a molecule expressed solely on the therapeutic cells. Including a marker protein in the genetic modification of therapeutic cells is one way of introducing a 'suicide' gene.

Several mechanisms for providing this safety method have been investigated, including the herpes simplex virus thymidine kinase (HSV-TK), an inducible caspase 9 (iCasp9), CD20 and the mutated human thymidilate kinase (mTMPK) enzyme (Marin et al. 2012). HSV-TK functions as a suicide gene because it confers sensitivity to ganciclovir; demonstrated both *in vitro* and *in vivo* (Tiberghien et al. 1994; Moolten et al. 1990). In a trial carried out by Bonini et al. (1997), using HSV-TK as a suicide gene was shown to be effective when adding ganciclovir to patients suffering from acute GvHD. In one patient, who was suffering from chronic GvHD, full sensitivity to ganciclovir was not demonstrated; transduced cells that were isolated *ex vivo* from the patient were shown to have unmodified sensitivity, suggesting that *in vivo* resistance can be conferred to genetically modified cells. Use of ganciclovir as a suicide gene also prevents patients with cytomegalovirus infections from receiving these cells.

iCasp9 was constructed by fusing a modified FK506 binding protein (FKBP) domain to the proteolytic domain of caspase 9 (Straathof et al. 2005). This molecule is non-immunogenic as it is derived from human molecules, except from one single amino acid substitution in the FKBP. In one study, in patients who developed GvHD, upon administration of a dimerising drug (AP103), apoptosis was induced and cells expressing the construct were eliminated. 90% elimination of modified T cells occurred within 30minutes of administration (Di Stasi et al. 2011). Another advantage over using HSV-TK as a suicide gene is that iCasp9 is not virus-derived. As a result, this should

prevent development of any memory T cells specific for the junctional sequences between the molecules in iCasp9, reducing the chance of the host immune system eliminating the modified T cells (Straathof et al. 2005).

mTMPK is a molecule that has been mutated to have a higher affinity for its substrate, the prodrug 3'-azido-3'-deoxythymidine-monophosphate (AZT-MP). By increasing the AZT concentrations, the rate of conversion of AZT-MP to AZT-triphosphate (AZT-TP) is increased, resulting in increased intracellular AZT-TP, which is cytotoxic (Sato et al. 2007). When tested alongside other suicide strategies, including CD20, HSV-TK and iCasp9, though, mTMPK is not as effective (Marin et al. 2012).

Use of CD20 as a suicide gene allows for elimination of CD20-expressing cells using the anti-CD20 mAb, rituximab. The main disadvantage of using this as a suicide mechanism is that it leads to the depletion of the patient's B cells as well as the genetically modified cells. In addition, modified cells cannot be used if the patient is undergoing rituximab treatment. Despite this, CD20 is promising as a suicide gene as the molecule allows for the sorting of modified cells and has also been shown to be non-immunogenic. Furthermore, the anti-CD20 mAb, rituximab, has been used previously in the treatment of B cell lymphomas and its role in the lysis of cells, through complement activation, has been well-characterised (Serafini et al. 2004).

1.4.9 Genomic manipulation to create a universal T cell

Gene modifications can be transferred into T cells via a variety of methods, split into three main categories – physical, chemical or biological. Physical methods include electroporation, ultrasound and biolistic particle delivery (Niidome & Huang 2002). Chemical methods use delivery agents such as calcium phosphate, polyethylenimine (PEI), and liposomes (Nishikawa & Huang 2001). Biological methods include viral vectors and transposons. Physical and chemical methods can be very efficient, but they are also transient, and stable integration and expression is only achieved in a small

number of cells (Sorrell & Kolb 2005). Electroporation, for example, requires applying an electric current to cells in order to allow entry of DNA into the cell. This disrupts the membrane and creates nm-scale holes in the membrane, through which the DNA can enter the cell. The drawback of this is that overall cell viability after electroporation drops and conditions have to be experimentally optimised for each cell line tested in order to achieve the most efficient protocol and increase cell viability.

As T cells have a rapid proliferation rate, it is vital to achieve gene transfer that is efficient and long-lasting. Currently, the best way to do this is to use retroviral or lentiviral vectors, which allow for stable and sustained expression of the transgene, as well as efficient integration into the genome. Retroviruses are able to insert their genome, containing the transgene of choice, into a distinct nucleotide sequence at non-specific sites throughout the host genome. Multiple studies have demonstrated the efficiency of retroviral integration into T cells, with most of these using Maloney murine leukaemia virus (SFG) derived vectors. Transduction efficiencies with these vectors can be 80-90% (Quintás-Cardama et al. 2007).

The issue of epigenetic silencing is one that affects retroviral integration, although it does not affect lentivirus, which appears to avoid this issue (Pannell & Ellis 2001). In addition, the integration site of the virus into the host genome cannot normally be predicted. As a result, integration may occur in a site that downregulates transgene expression, or integration may modulate activity of host genes at the integration site, potentially having damaging effects. Hacein-Bey-Abina et al. (2003) demonstrated this in two patients who developed leukaemia after having retroviral vector-based treatment for X-linked SCID (X-SCID). Genome analysis showed that the retrovirus had integrated near to the LIM domain only (LMO)-2 proto-oncogene promoter, leading to abnormal regulation and expression of LMO2. Site-specific integration of transgenes would therefore be an advantage, and is something that is achievable by other strategies, including zinc finger nucleases (ZFNs), meganucleases (MNs) and TALENs.

Due to the adverse events previously experienced, research focused on the safety of viral vectors. The long terminal repeats (LTRs), included in the vectors, contain elements that regulate and promote gene expression. LTRs contain enhancer and promoter regions and the transcription start and termination sites (Uren et al. 2005). This restricts efficient viral expression to the environments that match the conditions set by the viral LTRs. The Moloney MLV has tropism for T cells and B cells, which accounts for the high transduction efficiency that has been shown with this viral vector in previous T cell studies (Erkeland et al. 2003; Erkeland et al. 2004). Other efforts to improve retroviral safety include making the vectors self-inactivating (SIN) and to modify the promoter sequences to prevent read-through by improving the messenger ribonucleic acid (mRNA) termination and polyadenylation (Schambach et al. 2007). Retroviral gene therapy using the MLV vector has been successfully achieved in multiple animal models and clinical trials and it is this vector that is used in this project.

1.5 Safety

The main safety considerations in this project are the possibility of allorejection, GvHD, on-target off-site activity and SAEs. Some of the strategies tested in this project were designed to overcome these obstacles, and some of these potential risks were unable to be tested in this system, although they have been evaluated in other studies. Toxicity is the main issue to solve as, regardless of the efficacy of the therapeutic cells, if the toxicity is above acceptable thresholds, regulatory approval will be refused. These issues and potential solutions are discussed in the following sections.

1.5.1 Tumour lysis syndrome

Tumour lysis syndrome (TLS) is an unwanted side-effect of some effective therapies. It occurs when the contents of tumour cells are released into the

bloodstream, often as a result of tumour cell death that is caused by chemotherapy or immunotherapy. Due to this huge release of intracellular contents, the body's homeostatic systems and waste removal are quickly overloaded. This leads to electrolyte imbalances such as hyperuricemia, hyperkalaemia, hyperphosphatemia, and hypocalcaemia (Dhanraj & Biswajit 2014). These complications can lead to severe clinical consequences, including acute kidney injury, cardiac arrhythmias, pulmonary oedema, fluid overload, seizures, and death (Abu-Alfa & Younes 2010).

TLS occurs very soon after initiation of chemotherapy, within the first week. It most commonly occurs in patients suffering from haematological malignancies, especially those with high tumour loads. A model has been proposed in order to stratify patients with high, intermediate or low risks of TLS depending on the type of cancer present, the white blood cell (WBC) count and the type of therapy to be initiated. Patients with rapidly proliferating, therapy-sensitive tumours are among those at a higher risk of developing TLS (Coiffier et al. 2008).

When treating patients with universal T cells, the efficacy of the cells will need to be assessed in order to determine the risk of causing TLS. If the CAR expressed on the T cells is highly specific with a high affinity, it may cause rapid tumour cell lysis leading to TLS. Careful selection needs to be made to determine the optimal antibody affinity and efficacy. The relationship between antibody affinity and *in vivo* efficacy is an unclear one, with one example showing this. A study made on a panel of ERBB2 scFvs showed that those with a threshold affinity (Kd) of 10^{-7} to 10^{-8} M allowed specific localisation to the tumour. Uptake of the scFvs reached a plateau at a Kd of 10^{-9} to 10^{-11} M. From this panel, the scFv with the lowest affinity showed the optimal ability to distribute throughout the tumour. On the other hand, the scFv with the highest affinity localised mainly to the perivascular region of the tumour (Adams et al. 1998; Adams et al. 2001). Therefore, it can be seen that there needs to be a balance between affinity and tumour localisation in order to provide the most effective outcome, whilst also minimising the risk of inducing TLS. This is a key consideration needed when selecting the binding moieties of CARs to introduce into the therapeutic T cells.

1.5.2 Cytokine release syndrome

Cytokine release syndrome (CRS) is caused by a sudden increase in cytokines as a result of T cell engagement and proliferation. Mild CRS is the most common form prevalent in patients undergoing immunotherapy, with flu-like symptoms, fevers and myalgias. Severe CRS however, is characterised by symptoms including hypotension, pulmonary oedema, vascular leak and severe inflammatory syndrome, leading to multiple organ failure and possible death (Maude, Barrett, et al. 2014). Within these CRS categories, there is often large variability between patients, as the degree of cytokine elevation may not necessarily correlate to the severity of CRS or the response to therapy. Some patients display clinical symptoms that are not reflected in the sample analysis, and some sample analysis may suggest severe CRS yet the patient does not display the expected clinical symptoms (Klinger et al. 2012).

Clearly, the use of therapeutic T cells is designed to result in a degree of cytokine release. The difficulty is being able to manage the level of cytokine release and to be able to suppress it in case CRS develops. Corticosteroids are one of the main options for CRS management, having been shown to be effective in T cell activation, GvHD and inflammatory disorders (Martin et al. 2012). However, the use of steroids may result in a reduction in efficacy as previously seen (Porter et al. 2011).

Another approach may be to target the cytokines released during CRS. Regulators of innate and cell-mediated immunity include IL-6, IL-10 and IFNγ. IL-10 is a negative regulator and therefore not an ideal target for CRS treatment. Similarly, IFNy is released after T cell engagement, and is required for efficacy. IL-6 is an inflammatory cytokine involved in multiple processes including immune responses, bone and lipid metabolism (Mihara et al. 2012). Levels of IL-6 reach an apex during maximal T cell proliferation, and blockade of IL-6 by tocilizumab has been shown to reverse severe CRS in patients undergoing CAR T cell or blinatumomab treatment (Grupp et al. 2013; Teachey et al. 2013). There are other cytokines that could be targeted,

including MCP-1, MIP1B, IL2-R, IL1-R, IL-33, IL-13, IL-31 and TNFα, and some of these are currently being tested (Maude, Barrett, et al. 2014). Clearly there is a balance that needs to be found between efficacy and management of toxicity; reducing the risk of CRS induction whilst maintaining the highest possible efficacy.

1.5.3 Haemophagocytic lymphohistiocytosis

Haemophagocytic lymphohistiocytosis is characterised by fever, hepatosplenomegaly and cytopenia. It contains two main conditions, familial haemophagocytic lymphohistiocytosis (FHL) and secondary haemophagocytic lymphohistiocytosis (SHLH). FHL is an inherited disease, but SHLH can affect any age, and also subside spontaneously (Henter et al. 2002). Poorly controlled NK cells fail to stimulate apoptosis of activated cells, leading to a persistent inflammatory state, characterised by IL-6 and TNFα (Perez et al. 1984).

Secondary HLH has the same symptoms as primary HLH, but is caused by an activation of the immune system by underlying illness. If a reduction in HLH symptoms is not achieved by treatment of the underlying illness, then immunosuppressive chemotherapy is used, following the HLH-2004 protocol (Keith et al. 2012). This protocol involves the use of dexamethasone, VP-16, cyclosporine A, introthecal methotrexate and corticosteroids in order to reduce and alleviate the symptoms of secondary HLH (Henter et al. 2007). In many ways, it is similar to macrophage activation syndrome (MAS), which most commonly occurs in infants. Secondary HLH is an issue that will need to be taken into consideration for patients who are being treated and have underlying illnesses that may trigger HLH.

1.5.4 On-target, off-site activity

Tumour cells often display cell surface markers that are the same as, or very similar to healthy, non-cancerous cells. Some markers are down-regulated to prevent immune surveillance recognising the tumour cells, and others are up-regulated to block the induction of death signals. As previously stated, the selection of tumour target needs to be performed carefully. The molecule targeted by a CAR may be expressed on multiple cell types, some of which are healthy, others cancerous. Taking CD20 as an example, this is normally expressed on B cells, but is also expressed in B cell lymphomas. Targeting the CD20 molecule means that all of the B cells are targeted, not just the cancerous ones. Some previous work has been done to augment the expression of CD20 on tumour cells in order to increase killing with aCD20 antibodies. This study showed that IL-4, TNFα and GM-CSF caused an up-regulation of CD20 antigen expression (Venugopal et al. 2000).

Another example would be the NY-ESO-1 protein, which is in a class of cancer-testis (CT) antigens, which are expressed by some tumour types, but also in the testis. For TCR-modified T cells, the testis is not vulnerable to these T cells as the germ cells do not express MHC molecules (Morgan 2013). The NY-ESO-1 protein has been successfully targeted in ACT clinical trials, where objective clinical responses were observed in four of six patients with synovial cell carcinoma and five of eleven patients with melanoma tumours expressing the NY-ESO-1 antigen (Robbins et al. 2011).

A final example is CD44, which is expressed widely in haematological and epithelial tumours. The CD44v6 isoform has been demonstrated to be expressed on the majority of AML and multiple myeloma (MM) tumour cells. A CAR targeting CD44v6 was developed and shown to inhibit tumour formation of AML and MM cells in xenograft models. What was also discovered was that CD44v6 is also expressed on keratinocytes and circulating monocytes. Keratinocytes were not recognised by the aCD44v6 CAR, but monocytes were, causing monocytopenia in the animal models tested. As a result, this study added an iCasp9 suicide gene to the therapy, allowing for depletion of the therapeutic cells in order to manage long term monocytopenia (Casucci, Robilant, et al. 2013). This is a very clear example of on-target, off-site toxicity. In many cases, it may be unavoidable, but with correct planning of therapy and

detailed analysis of the target, severe effects can be planned for and prevented, or responded to quickly.

1.6 Project Aims

The aim of this project is to investigate ways in which these strategies can be collated in order to develop a universal cell product (third party), where one T-cell pool could be used to treat many patients. This would allow many more patients to be treated and for treatment to be commenced in a shorter time frame. The main two barriers to this are: (1) rejection of the adoptively transferred allogeneic T-cells by the host immune response (alloreactivity); (2) rejection of the host by the adoptively transferred T-cells, causing GvHD. The first barrier can theoretically be overcome by knock-down of HLA in adoptively-transferred allogeneic T-cells. This may result in an additional obstacle of NK-mediated null cell deletion. This may be overcome with HLA-G expression, mimicking the situation of trophoblast invasion in pregnancy (Rouas-Freiss, Gonçalves, et al. 1997; Rouas-Freiss, Marchal, et al. 1997). This was demonstrated by a lack of cell killing by the T cell leukaemia NK-like YT2C2 clone when interacting with a HLA-negative cell line (K562), expressing HLA-G1 and HLA-G2 (Rouas-Freiss, Marchal, et al. 1997). GvHD can be overcome by TCR knockdown and re-directing the T-cell specificity using a CAR. A sort-suicide gene can be introduced to allow clinical grade sorting of the modified cells, and as a mechanism by which modified cells can be depleted from the patient in case of adverse events.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Reagents

2.1.1.1 General reagents and chemicals

14ml Falcon round-bottom tube	BD	352006
(methyl-^{3}H) Thymidine	Perkin Elmer	NET027001MC
^{51}Chromium labelled sodium chromate	Perkin Elmer	NEZ030002MC
Agarose	Bioline	BIO-41025
Boric acid	VWR International	20185.360
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A7906
Carbenicillin	VWR International	69101-3
Ethidium Bromide	BDH Chemicals	443922U
Ethylenediaminetetraacetic acid (EDTA) Disodium salt	VWR International	20302.260
Electroporation Cuvettes, 2mm gap	BTX	45-0125
Filtermat	Perkin Elmer	1450-421
Glycerol	Sigma-Aldrich	G5516
Haemocytometer	Hirschmann Laborgeraete GmbH	8100103
LB Medium	MP Biomedicals LLC	3002-031
LB-Agar Medium	MP Biomedicals LLC	3002-231
LS Columns	Miltenyi Biotec	130-042-401
MeltiLex solid scintillator	Perkin Elmer	1450-441
MS Columns	Miltenyi Biotec	130-042-201
Nalgene® Mr. Frosty®	Thermo Scientific	5100-0001
Parafilm	VWR International	291-1212
PBS tablets	Oxoid	BR0014G
PFA 4% in PBS	Insight Biotechnology Ltd	sc-281692
Plate seals	Perkin Elmer	1450-462
Propidium Iodide	Sigma-Aldrich	P4170
TALEN mRNA	Cellectis Bioresearch	
Terrific broth	Merck Chemicals	1.01629.0500
Tris base	Fisher Scientific	BPE152-5
Trypan Blue solution	Sigma-Aldrich	T8154

2.1.1.2 Molecular cloning enzymes, reagents and kits

Hyperladder DNA ladder	Bioline Limited	BIO-33026
Murine Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer	Qiagen	QT01658692
Nucleobond Midiprep kit	Thermo Scientific Abgene	NZ74041050
Nucleospin Gel and PCR Clean up kit	Thermo Scientific Abgene	NZ740609250
Nucleospin Miniprep kit	Thermo Scientific Abgene	NZ740588250
Oligo primers	Integrated DNA technologies (IDT)	
Phusion DNA polymerase	New England Biolabs UK Ltd	M0530L
Platinum PCR SuperMix	Invitrogen	12532-016
Restriction endonucleases & buffers	New England Biolabs UK Ltd	(various)
TOPO TA cloning kit	Invitrogen	45-0641
Qiagen Gel extraction kit	Qiagen	28706
Qiagen Miniprep kit	Qiagen	27106
QIAquick PCR purification kit	Qiagen	28104
Quick Ligation kit	New England Biolabs UK Ltd	M2200L

2.1.1.3 Bacteria

New England Biolabs (NEB) 5-α Competent E. coli (High Efficiency)	New England Biolabs UK Ltd	C2987H
NEB 5-α Competent E. coli (Subcloning Efficiency)	New England Biolabs UK Ltd	

		C2988J

2.1.1.4 Tissue culture plastic-ware

0.2µM pore syringe filter	Sartorius	16532K
0.45µM pore syringe filter	Sartorius	16537K
100mm tissue culture (TC) dishes	Corning	430167
6 Well plate TC treated	Corning	3506
12 Well plate TC treated	Corning	3513
24 Well plate TC treated	Corning	3524
24 Well plate non-TC treated	BD Biosciences	351147
48 Well plate TC treated	Corning	3548
96 Well plate flat bottom	Corning	3596
96 Well plate V-bottom	Greiner Bio-One	650-180
96 Well plate round-bottom	Corning	3799
15ml Falcon tubes	Corning	430791
50ml Falcon tubes	Corning	430829
250ml centrifuge tubes	Corning	430776
6.5mm Transwell, 0.4µM pore	Corning	3470
Flask Ez Nunclon 175cm	Thermo Electron	159910
Flask Ez Nunclon 75cm	Thermo Electron	156499
Flask Ez Nunclon 25cm	Thermo Electron	156367

2.1.1.5 Tissue culture medium and supplements

Anti-CD28 antibody good manufacturing practice (GMP) pure		
	Miltenyi	170-076-117
Anti-CD3 (OKT3) GMP pure	Miltenyi	170-076-124
Anti-CD34 microbeads (human)	Miltenyi Biotec	130-046-702
Anti-CD56 microbeads (human)	Miltenyi Biotec	130-050-401
Cell dissociation medium	Sigma-Aldrich	C5914
Cryopreservation Medium	Lonza Bio Science Ltd	

		US12-132A
Cytoporation medium T buffer	BTX	47-0002
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
Dulbecco's PBS (DPBS) solution	Invitrogen Life Technologies	
		14190169
Dynabeads - Human CD3/CD28	Life Technologies	11131D
EDTA solution (0.5M)	Sigma-Aldrich	E7889
Foetal calf serum (FCS)	Biosera	S1900/500
Ficoll-Paque	GE Healthcare Life sciences	
		17-1440-03
Genejuice	Merck Chemicals Ltd	
		70967-3
GlutaMAX	Invitrogen Life Technologies	
		35050087
IL-2 Recombinant human	Genscript	Z00368
Iscove's Modified Dulbecco's Media (IMDM)		
	Lonza Bio Science Ltd	
		BE12-726F
Normocin	InvivoGen	ant-nr-1
Phytohaemagglutinin (PHA)	Sigma-Aldrich	L9017
Polybrene	Millipore	TR-1003-G
Retronectin	Lonza Bio Science Ltd	
		T100B
Roswell Park Memorial Institute (RPMI)	Lonza Bio Science Ltd	
		BE12-167F
Trypsin-EDTA solution	Sigma-Aldrich	T4049

2.1.2 Buffers and solutions

All buffers and solutions, unless otherwise stated, were prepared in deionised, reverse osmosis filtered laboratory grade water. They were also either

sterilised in an autoclave at 121^{o}C for 15minutes or sterile filtered through a 0.22µM filter using a vacuum. Recipes for buffers are below:

Table 2 *– Buffer composition, ingredients and recipes*

Buffer	**Composition and ingredients**
Flow cytometry fixing buffer (FACS fixing buffer)	FACS buffer + 0.5% Paraformaldehyde (PFA) (Insight)
Luria-Bertani (LB) broth	LB tablets/powder were dissolved in deionised laboratory grade water according to manufacturer's recipe (MP Biomedicals LLC)
MACS/FACS buffer	1% FCS in PBS
Phosphate buffered saline (PBS) (PAA Laboratories Ltd H15-002)	PBS tablets (Oxoid) were dissolved into 1L of deionised laboratory grade water
Propidium iodide (PI) stock solution	25mg PI (Sigma) was suspended into 10ml of deionised laboratory grade water to establish a 2.5mg/ml stock solution
Terrific Broth (TB)	Terrific broth tablets/powder (Merck) were dissolved in deionised laboratory grade water, with glycerol (Sigma-Aldrich) added as required
Tris/Borate/EDTA buffer (TBE) (x10)	9.3g of EDTA, 55g of Boric Acid and 108g of Tris-base was dissolved into 1L of deionised laboratory grade water

2.1.3 Antibodies

The following antibodies were used during FACS analysis and in blocking assays:

2.1.3.1 General conjugates

Streptavidin-APC	AbD Serotec	STAR119
Streptavidin- Phycoerythrin (PE)	eBioscience	12-4317-87
Streptavidin-PE-Cyanine-7 (Cy7)	eBioscience	25-4317-82
Streptavidin- Peridinin chlorophyll (PerCP)	BD Biosciences	554064

2.1.3.2 Human antibodies

Dilutions used according to manufacturers' guidelines

CD33-APC	eBioscience	17-0338
CD34-(581)-APC	BD Biosciences	555824
CD34-(QBEnd10)	Dako UK	M7165
CD34-(QBEnd10)-Biotin	AbD Serotec	MCA547B
CD34-(QBEnd10)-PE	Abcam	ab30377
CD3-APC	BD Biosciences	555335
CD3-PE	Beckman Coulter	PN IM1282
CD3-PerCP	BD Biosciences	552851
Goat-anti-human-Fc-Dylight488	Jackson ImmunoResearch	109-485-098
Goat-anti-human-Fc-DyLight649	Jackson ImmunoResearch	109-496-127
HLA-A/B/C-APC	BD Biosciences	555555
HLA-A2-Fluorescein isothiocyanate (FITC)	BD Biosciences	551285
HLA-DR-APC	BD Biosciences	559868
HLA-G-PE	eBioscience	12-9957-41
HLA-G-PerCP	eBioscience	46-9957-41
IFNγ-FITC	eBioscience	11-7319
TCR-Biotin	eBioscience	13-9986
TCR-FITC	BD Biosciences	555547

TCR-PE	BD Biosciences	555548
BD Cytofix/Cytoperm Fixation/Permeabilisation solution kit with BD GolgiStop	BD Biosciences	554715
Paraformaldehyde (PFA)	Insight	10351641

2.1.3.3 Blocking antibodies

HLA-Class I (W6/32 Hybridoma)	Dako	M073601-2
HLA-Class II (CR3/43 Hybridoma)	Dako	M077501-2

2.1.4 Cell lines

293T	Human embryonic kidney (HEK) cell line – European collection of cell cultures (ECACC)-05/B/013).
Jurkat	Human acute lymphoblastic leukaemia T cell line derived from non-Hodgkin lymphoma patient. Kind gift from Dr Cliona Rooney, Centre for Cell and Gene Therapy, Houston, TX, USA.
TCRko Jurkat	Derived from a patient with T cell leukaemia, demonstrated to lack endogenous TCR expression of both chains. Obtained as a kind donation from Prof Hans Stauss, Royal Free Hospital, London, NW3 2PF.
SupT1	T lymphoblast cell line derived from a patient with T cell lymphoblastic leukaemia (Baylor College of Medicine).
K562	Undifferentiated granulocytic cell line derived from a patient with chronic myelogenous leukaemia (ECACC).

BBM.1 (HB-28) Murine hybridoma cell line derived from mouse spleen cells and P3X63Ag8 myeloma cells (ATCC HB-28)

2.1.5 Media recipes

Table 3 *– Tissue culture medium recipes*

Medium	**Recipe**
Complete IMDM (Lonza BE12-726F)	10% FCS (Biosera), 1% GlutaMAX (Invitrogen)
Complete RPMI (Lonza BE12-167F)	10% FCS (Biosera), 1% GlutaMAX (Invitrogen)
Freezing media with 15% DMSO (Lonza 12-132A)	18ml of FCS (Biosera) was added to this medium to generate a solution with 15% FCS and 13% DMSO
PBMC assay medium	Complete RPMI as above, supplemented with 50 I.U/ml of recombinant human IL-2 (Genscript)

2.1.6 Equipment

AGO HS MP-1	X-ray irradiator
BD Biosciences	LSR Fortessa cell analyser
BD Biosciences	FACSVerse flow cytometer
Beckman Coulter	CyAn ADP flow cytometer
BioRad	Peltier Thermal Cycler
LKB Wallac	1282 Compugamma Gamma Counter
Nanodrop®	ND-1000 Spectrophotometer
Perkin Elmer	1450 MicroBeta TriLux Microplate

Scintillation and Luminescence Counter

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Small scale DNA preparation

Small scale (miniprep) DNA preparation was as follows: Single colonies were picked from an agar plate and grown overnight in 14ml Falcon tubes at 37^{o}C with 200-250rpm shaking, in 4-5ml LB supplemented with 100µg/ml Carbenicillin. The Qiagen or Macherey-Nagel miniprep kit was used to isolate plasmid DNA according to the manufacturer's instructions. Constructs were verified by restriction digest followed by agarose gel analysis and/or DNA sequencing.

2.2.1.2 Large scale DNA preparation

Large scale (midiprep) DNA preparation was performed as follows: 50-100ml of TB, supplemented with 100µg/ml of Carbenicillin, was inoculated with a 5ml bacterial culture and grown overnight at 37^{o}C with 200-250rpm shaking. The Qiagen or Macherey-Nagel midiprep kit was used to extract plasmid DNA from the resulting bacterial prep, following the manufacturer's instructions. Midiprep DNA was verified by multiple restriction digests to provide a clearly identifiable band pattern on an agarose gel.

2.2.1.3 Measurement of DNA concentration

DNA concentration was calculated by measuring the absorbance of light with a wavelength of 260 nm (A260) using a NanoDrop ND-1000

spectrophotometer with a 0.2mm pathlength; at this wavelength 50µg/ml of double-stranded DNA has an absorbance of 1. The ratio of absorbance at 260nm:280nm can be used to establish DNA purity. A ratio of 1.8 indicates a high level of DNA purity with little or low levels of RNA and/or protein contamination.

2.2.1.4 Splicing of DNA fragments by overlapping extension PCR

Transgenes for cloning into expression vectors were generated by overlapping extension PCR. Transgenes were designed into multiple fragments with an *NcoI* restriction endonuclease site designed in the 5' end of the N-terminal fragment, along with a signal peptide, and a *MluI* restriction endonuclease site designed into the 3' end of the C-terminal fragment. Oligonucleotides, complimentary to the fragment being amplified, were used to generate the genes and assembled by overlapping PCR as follows: Oligonucleotide stocks were diluted to 25µM before being added to PCR tubes containing dH_2O, HF Buffer, deoxyribonucleotide triphosphates (dNTPs), and Phusion Hot-Start II Polymerase (Finnzymes 2013). The primary PCR was used to amplify the fragments and was run on a Peltier Thermal Cycler (BioRad) as follows: 98^oC for 2 minutes, 98^oC for 40 seconds, 65^oC for 40 seconds, 72^oC for 1minute per kb of amplification, cycled back to the second step 35 times and then 72^oC for a final 10 minutes before the reaction was complete. Each reaction was run in a separate lane by gel electrophoresis, as described in 2.2.1.6 Gel electrophoresis, and extracted into 30µl of dH_2O using a Qiagen gel extraction kit as described in 2.2.1.7 Gel extraction. For the secondary PCR, 3.5µl of each primary PCR reaction was added to the same mix as the primary PCR, with a 3x volume. The PCR was repeated as above on the thermal cycler, with an increased time for extension according to the length of the DNA being fused. The PCR products were run on an agarose gel and desired bands were extracted into 80µl of dH_2O using the Qiagen gel extraction kit. This product was then digested with the appropriate restriction enzymes (2.2.1.9 Restriction endonuclease digestion), ligated into the relevant linearised vector (2.2.1.10

Ligation) and transformed into competent *E.coli* (2.2.2.2 Bacterial transformation). Verification restriction enzyme digests were performed and run on an agarose gel to select clones to be sent for capillary sequencing by Scientific Support Services (in-house).

2.2.1.5 Site-directed mutagenesis using oligonucleotide primers

In the instance of HLA-G extraction and cloning from genomic DNA, correct assembly of the gene was not completely successful. As a result, there were some mutations in the sequence that would have prevented, or possibly hindered, optimal expression as a transgene. In order to rectify this, oligonucleotide primers were designed over the relevant mutations that would revert those mutations back to the original HLA-G sequence. Full length, mutated HLA-G was used as a template, and oligonucleotides were diluted to a concentration of 25µM before being used in a PCR reaction as described in the PCR set-up in 2.2.1.4 Splicing of DNA fragments by overlapping extension PCR

2.2.1.6 Gel electrophoresis

The size of PCR products and digested plasmids was confirmed by gel electrophoresis. 1% agarose gels were prepared in 1x TBE buffer by boiling in a microwave. Once melted, 0.5µg/ml ethidium bromide was added to allow DNA visualisation. DNA samples were mixed with loading buffer at a ratio of 5:1 prior to addition to set agarose gels. Relevant DNA hyperladder was included to allow determination of DNA fragment size. Gels were electrophoresed at 50-120V and up to 150mA in 1x TBE buffer until adequate separation of bands was observed. Fragments were visualised by exposure to UV light using a UV gel documentation system. PCR fragments requiring further manipulation were visualised using a blue light box to prevent DNA damage as a result of ultraviolet (UV) light exposure. Relevant bands were

excised from the gel using a sterile scalpel before DNA gel extraction as described in 2.2.1.7 Gel extraction.

2.2.1.7 Gel extraction

Following identification of the correct bands by gel electrophoresis, the desired band was visualised using a blue light box and excised from the gel using a clean scalpel blade. Gel extraction was then performed using the Qiagen QIAquick or Macherey-Nagel gel extraction kit according to manufacturer's instructions.

2.2.1.8 PCR purification

The product of PCR reactions was purified with the Qiagen QIAquick or Macherey-Nagel PCR clean-up kit according to the manufacturer's instructions. A portion of the resultant product was examined by gel electrophoresis to confirm size and purity. Products were subsequently digested by restriction enzymes as described in 2.2.1.9 Restriction endonuclease digestion before downstream processing.

2.2.1.9 Restriction endonuclease digestion

A 20-fold over-digestion was performed to produce fragments for subsequent ligation or for confirmation of successful cloning. 0.4-5µg of plasmid DNA was digested in a final volume of 30-100µl. The manufacturer's recommended buffer was used, diluted to 1x with ddH_20 and 0.1mg/ml BSA was added when needed. The volume of enzyme varied according to concentration but never exceeded 10% (v/v) of the final reaction volume. Digestion was performed for 2 hours at 37^{o}C and verified by agarose gel electrophoresis as described in 2.2.1.6 Gel electrophoresis. Double digestions were performed in parallel where

buffers were compatible or sequentially following clean-up of DNA where buffers were incompatible using the relevant clean-up kit. For digestion reactions that ran overnight, the heating block was programmed to heat-inactivate an enzyme once completed, or to reduce the temperature to 4^{o}C if the enzyme could not be heat-inactivated. To generate vector backbone, or vector derived insert fragments, 5µg of plasmid vector DNA was used. Genes generated as above were subcloned as 'sticky-end' fragments into the relevant vector backbone as described in 2.2.1.10 Ligation.

2.2.1.10 Ligation

Following restriction enzyme digestion of vector and insert, ligations were performed using 100ng of vector DNA at a vector:insert molar ratio of 1:4-1:8, depending on insert size and concentration. Ligations were performed at room temperature for 5 minutes using Quick Ligase (NEB) according to the manufacturer's instructions. 2µl of the ligated DNA was transformed into high efficiency C2987 chemically competent *E.coli* bacteria as described in 2.2.2.2 Bacterial transformation.

2.2.1.11 Plasmids

The retroviral SFG plasmid vector (Büeler & Mulligan 1996) was used for expression of all genes generated in this project. SFG is based on the murine Moloney Leukaemia Virus, with the transgene start codon at the start site of the deleted viral *env* gene. This is followed by an internal ribosomal entry site (IRES), allowing expression of the relevant downstream reporter gene (if included). Fragments were cloned in using a unique restriction enzyme site upstream of the transgene start site, and the unique restriction enzyme site immediately following the transgene termination codon. For constructs containing the '2A' sequence, the dual construct was translated as a single peptide, with separation of the separate components achieved by the self-

cleaving activity mediated by the TaV (Thosea asigna virus) 2A sequence resulting in theoretical equimolar expression of both transgenes. Constructs denoted SFGmR also include a scaffold attachment region to enhance transgene expression.

2.2.1.12 Codon optimisation and gene synthesis by oligonucleotide assembly

An optimal sequence was designed using pMol software written by Dr Martin Pule. This allowed gene design based on optimisation of human codon usage to allow for optimal gene expression, raising GC content to 70%, reducing local sequence repeats, local hairpins, to a minimum and avoiding splice signals. The sequence was assembled with ligation-by-PCR using Phusion polymerase from overlapping oligonucleotides. Overlapping oligonucleotides were designed to allow short sections of DNA to overlap as demonstrated in **Figure 1**. Using these oligonucleotides as templates, two rounds of DNA amplification were performed. The first round produced short sections of template DNA and the second round allowed for amplification of the desired sequence using the terminal oligonucleotides.

Figure 1 – Gene construction by oligonucleotide assembly. Overlapping oligonucleotides (in blue) are used to generate the full length gene shown by the nucleotide sequence.

2.2.1.13 Trizol®-based RNA isolation

Around 5x10^6 PBMCs isolated by Ficoll extraction were re-suspended in 1ml of Trizol® reagent, with pipette agitation to achieve generation of a single cell suspension to allow for effective cell lysis. Cells were incubated in Trizol® for 5minutes at room temperature before being transferred to a sterile Eppendorf tube. 200µl of chloroform was added to the Trizol® lysate. This suspension was vigorously vortexed for 15seconds, incubated at room temperature for 3minutes then centrifuged at 11,500G for 15minutes. The upper aqueous layer was removed and transferred to a fresh sterile Eppendorf tube. 500µl of isopropanol was added and inverted twice before incubation at room temperature for 10minutes. This sample was centrifuged at 11,000G for 10minutes at 4^{o}C. The supernatant was removed, and the pellet was washed twice with 1ml of 75% ethanol, each time the sample was re-pelleted by centrifugation at 7,000G for 5 minutes at 4^{o}C. Following the second wash, the ethanol supernatant was aspirated by pipette and the Eppendorf tube was dried in a sterile hood for 20minutes. The RNA sample was then re-suspended in 40µl of molecular biology grade water and the concentration was assessed by spectrophotometric measurement by Nanodrop prior to cDNA generation.

2.2.1.14 cDNA generation

cDNA was generated from a cellular RNA sample by a two-step process. In the first step, a 20µl reaction mixture composed of 1µg of RNA, 2µl of Random Decamers, 4µl of 2mM dNTPs, with the remaining volume made up to 20µl by molecular biology grade water. This reaction was heated at 70^{o}C for 3minutes before being transferred to ice for 1minute. 2µl of 10x reverse transcriptase buffer was then added to the above reaction mixture followed by 1µl of RNasin and 1µl of reverse transcriptase. This second reaction was then incubated for 1 hour at 42^{o}C, followed by a 5minute incubation at 95^{o}C to deactivate the polymerase enzyme. Confirmation of successful cDNA generation was achieved by amplification of the GAPDH housekeeping gene. Forward and reverse primers for the human GAPDH sequence (GCCGAGCCACATCGCTCAGA, GAGGCATTGCTGATGATCTTG

respectively) were kindly supplied by Prof. Rosemary Gale. Primers for murine GAPDH amplification (Qiagen) were generously supplied by Dr. Jenny McIntosh.

2.2.1.15 Isolation of heavy and light chains from hybridoma

Following successful RNA extraction and cDNA generation from hybridoma cells, primers for heavy and light chain amplification were obtained from IDT according to the protocol by Toleikis et al. (2004) and amplified using the platinum PCR SuperMix high fidelity kit (Invitrogen) which employs *Taq* polymerase for amplification, selected to facilitate our subsequent TOPO-TA cloning strategy. Agarose gel electrophoresis was performed to determine successful heavy and light chain amplification. These were then TOPO-TA cloned as per manufacturer's instructions (Invitrogen).

2.2.1.16 TOPO-TA cloning

To facilitate screening of putative hybridoma cDNA fragments, a TOPO-TA (Invitrogen) cloning strategy was used to identify successful isolates. The supplied TOPO plasmid vector is supplied in a linearized format possessing a single 3'-thymidine (T) overhang with the *Topoisomerase I* enzyme covalently linked to the vector. As the *Taq* polymerase demonstrates a non-template-dependent terminal transferase activity, *Taq*-enzyme PCR amplicons possess a 3'-terminal deoxyadenosine (A) overhang. The presence of the corresponding T-A overhangs and the *Topoisomerase I* enzyme facilitates efficient ligation of *Taq*-amplified PCR products into the linearized vector as illustrated by **Figure 2**. Following ligation, high-efficiency *E. coli* (NEB) were transformed with the TOPO vector and plated onto Carbenicillin infused agar plates pre-coated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Further discrimination of successful vector integrants is achieved through the chromogenic identification facilitated by deletion of galactosidase enzyme

activity. X-gal is a lactose analogue which will form an indigo pigmented substrate due to dimerization of 5-bromo-4-chloro-3-hydroxyindole following galactosidase-mediated cleavage. Where the galactosidase enzyme sequence of the TOPO vector is interrupted by PCR ligation, enzymatic activity is ablated and resultant colonies will appear white on the agar plate. Following overnight culture, white colonies located proximal to indigo colonies were isolated for further analysis.

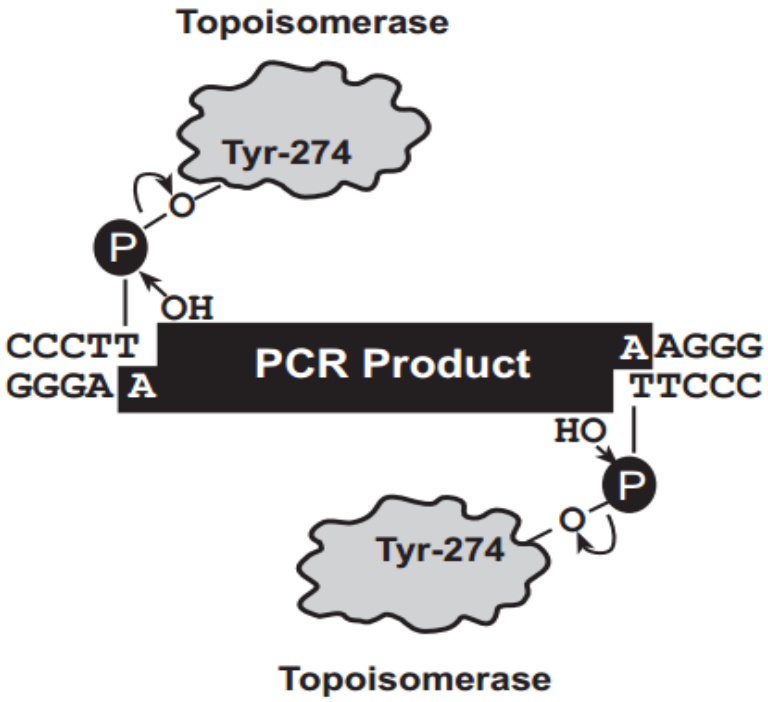


Figure 2 – TOPO-TA cloning strategy. Illustration is taken from the Invitrogen TOPO-TA cloning manual

2.2.2 Bacterial manipulation

2.2.2.1 Growth and maintenance of E.coli

E.coli were grown in liquid LB (miniprep) or TB (midiprep) media with 10mg/ml Carbenicillin (LB-Carb/TB-Carb) at 37^{o}C with agitation at 200-250rpm, or streaked out on solid LB-Carb agar plates. For long term storage, bacterial

cultures grown from a single colony were stored in 20% volume for volume (v/v) glycerol and stored at -80ºC.

2.2.2.2 Bacterial transformation

Competent DH5α *E.coli* were transformed by heat shock as follows: 25µl competent bacteria were thawed on wet ice. For DNA grow-up, C2988 *E.coli* were used; for ligations, C2987 *E.coli* were used. 1-10ng DNA or ligation reaction mix was added to the relevant *E.coli* and incubated on ice for 30minutes. Bacteria were transferred to a 42ºC water bath for 35 seconds then placed on ice for 2minutes. 250µl SOC media was added and the cells were incubated at 37ºC with 200-250rpm shaking for 40-80minutes. The resulting culture was streaked onto an LB-Carb agar plate (100µg/ml) and incubated overnight at 37ºC in a bacterial incubator.

2.2.3 Cell culture

2.2.3.1 Suspension cells

Non-adherent suspension cell lines, including SupT1s, Jurkats and TCRko Jurkats were cultured in complete RPMI. K562s were cultured in complete IMDM. All of these cell lines were cultured in upright 75cm^2 or 175cm^2 tissue culture flasks at 37ºC and 5% CO_2. Cells were maintained at a density of 0.2-2x10^6/ml; when confluent they were passaged at a dilution of 1:8-1:20 by removal of cells and old media followed by addition of fresh complete media. Discolouration of the phenol red indicator was used to visualise cell confluency.

2.2.3.2 Adherent cells

Adherent cells, including 293T cells, were cultured in complete IMDM in 175cm^2 flasks at 37^{o}C and 5% CO_2. When the cells reached around 80% confluency, they were washed in 1x PBS before adding trypsin/EDTA solution. Following dissociation treatment, the cells were re-suspended in a single cell solution, centrifuged at 400G for 5minutes and the media was discarded. Cells were then re-suspended in fresh complete IMDM and re-plated in a fresh flask at a dilution of 1:8-1:12.

2.2.3.3 Hybridoma cell lines

BBM.1 hybridoma cells were ordered as a cryopreserved sample from American Type Culture Collection (ATCC) and were recovered upon delivery as described in section 2.2.3.4 Cryopreservation and recovery of cryopreserved cells. Cells were initially cultured at 37^{o}C and 5% CO_2 in complete RPMI at a density of 2x10^5/ml and subsequently maintained at 1x10^5-1x10^6 cells/ml as described in the culture instructions (LGC 2012).

2.2.3.4 Cryopreservation and recovery of cryopreserved cells

For long-term storage, where appropriate, cells were cryopreserved as follows: Cells were harvested whilst in optimal growth conditions and counted before being centrifuged. The media was removed and the cell pellet was left on ice for 10minutes. Cryopreservation tubes were also cooled on ice. Cells were then re-suspended at 5x10^6/ml in freezing media. 1ml of the freezing media-cell suspension was aliquoted to each cryopreservation tube. Cells were then transferred to -80^{o}C in a cooled isopropanol freezing container (“Mr. Frosty” Nalgene), allowing for a controlled temperature decrease of around -1^{o}C per minute. The following day, cells were transferred to the vapour phase of liquid nitrogen for long term storage in a liquid nitrogen dewar. When required, cells were removed from liquid nitrogen, rapidly warmed to 37^{o}C and

washed twice in warm complete media before being cultured as previously described for downstream applications.

2.2.3.5 Isolation of PBMCs

Consent was gained before obtaining any blood from healthy donors, with a trained medic performing the venesections. Donors were monitored before and after venesection to ensure safety at all times. No more than 100ml was taken from any donor at one time. Whole blood obtained by venesection and anti-coagulated with sterile EDTA was diluted 1:1 with plain RPMI and 25ml was layered onto 12.5ml Ficoll-Paque in 50ml centrifuge tubes. Tubes were centrifuged at 750G for 40 minutes at room temperature without brake. After centrifugation, the buffy coat at the Ficoll-plasma interface was removed using a sterile transfer pipette. The PBMCs were then washed twice; once in plain RPMI; the second time in complete RPMI, before being re-suspended in complete RPMI. Cells were then either cryopreserved (see section 2.2.3.4 Cryopreservation and recovery of cryopreserved cells) or cultured at $1x10^6$/ml in complete RPMI in 24-well tissue culture treated plates. For transduction experiments, PBMCs were stimulated on the day of isolation with 5µg/ml PHA, followed by 100IU/ml recombinant human IL-2 on day 2, ready for transduction 24hours later.

2.2.3.6 HLA Typing

PBMCs were HLA-typed in one of two ways. Cells were either stained with the relevant subset(s) of anti-HLA antibodies (see *2.2.6 FACS analysis*), or cells were submitted for HLA-typing to the Bone Marrow Transplant Office at Great Ormond Street Hospital, London, where PBMCs were analysed for HLA type by sequence-specific oligonucleotide primed PCR (PCR-SSO).

2.2.3.7 Alternative T cell stimulation

The most commonly used method of stimulation of T cells was using the PHA/IL-2 protocol described above. For T cells that were to be electroporated with mRNA, alternative strategies were tested in order to try and optimise T cell stimulation. One method attempted was to use CD3 (OKT3) and CD28 antibodies in place of PHA, but to still use IL-2 in the same way as described above. CD3/CD28 Dynabeads (Life Technologies) were as another option, which could then be removed by MACS columns if required, according to 2.2.7.1 General magnetic bead selection protocol.

2.2.3.8 Electroporation of mRNA into T cells

T cells were stimulated in one of the ways described above for three days after Ficoll-Paque isolation from PBMCs. On day three, T cells were harvested and re-suspended in cytoporation medium T buffer (BTX) at a concentration of $2.5x10^6$/ml. 400µl of T cells were added to 2mm electroporation cuvettes (BTX), along with 5-10µg of mRNA. A control mRNA encoding the eGFP marker gene was included. T cells were electroporated according to the following protocol:

Table 4 – Electroporation protocol for electroporation of human T cells with mRNA

Pulse	Group 1	Group 2	Group 3
Amplitude (V)	1200	1200	130
Duration (ms)	0.1	0.1	0.2
Interval (ms)	0.2	100	2
Number of pulses	1	1	4

After electroporation, T cells were recovered into pre-warmed complete RPMI for 24-48hours at 37^{o}C, 5% CO_2 without cytokines. Cells were recovered in this way to prevent stimulation through the TCR, which would help TCR^+ T cells to survive preferentially over the TCR^- T cells. It also takes at least

24hours for the mRNA to be translated and expressed and for remaining TCRs on the cell surface to be internalised. Modified T cells were transduced with retroviral supernatant containing a CAR using the method described in section 2.2.5 Retroviral transduction.

2.2.4 Retroviral work

2.2.4.1 Retronectin coating of TC plates

Non-tissue culture treated 24-well plates were prepared by pre-coating plates with 500µl of PBS supplemented with 8µl of retronectin/ml at least 24hours prior to suspension cell transduction. Plates were wrapped in Parafilm and stored at 4^{o}C until required. Retronectin supplemented PBS was reused up to twice by direct transfer to fresh plates and stored as above until required.

2.2.4.2 Transient transfection for expression testing

Transient transfections were performed in multi-well TC-treated dishes or plates as required with volumes of media, Genejuice and DNA as indicated by **Table 5**:

Table 5 *– Transfection calculation matrix*

Plate / dish	Genejuice	Plain media	DNA
100mm dish	30µl	470µl	12.5µg
6-well plate	5µl	95µl	2µg
12-well plate	2.5µl	47.5µl	1µl
24-well plate	1.25µl	23.75µl	0.5µg

2x10^{6} HEK293T cells were plated into 6-well plates, 2ml cell culture/well, 24hours prior to transfection. Cellular confluence was assessed by microscopic examination to ensure optimal density prior to transfection.

A bulk transfection mixture was prepared where 5µl of Genejuice (Merck) was added to 95µl of plain RPMI for each transfection condition to be tested. Genejuice is a lipid-based transfection reagent that complexes with DNA to facilitate transport of DNA into cells during transfection. Following a 5minute incubation to allow formation of Genejuice-micelles, 100µl of the transfection mixture was transferred to a separate well of a round-bottomed 96-well plate. 2µg of plasmid DNA was used for each separate condition to be tested. Following a 15minute incubation of DNA, transfection was performed by drop-wise addition of the transfection mixture over the 293T cell culture.

2.2.4.3 Generation of retroviral supernatant

$1.5x10^6$ 293T were plated per 10cm tissue culture dish the day prior to transfection in 10ml complete IMDM. For retroviral supernatant production, RD114-pseudotyped transient retroviral supernatant was generated by triple transfection of 4.69µg Peq-Pam plasmid (Moloney GagPol), 3.125µg RDF plasmid (RD114 envelope), and 4.69µg SFG (or relevant) plasmid, per 10cm plate, into 293T cells using GeneJuice (Novagen). DNA was combined in 470µl plain IMDM and 30µl Genejuice was added. Transfection mix was incubated for 15 minutes at room temperature, followed by drop-wise addition to cells. Supernatant was harvested at 48hours and stored at 4^{o}C overnight. 10ml of pre-warmed complete IMDM was added to the plates and a second harvest was collected at 72hours. The two harvests were combined before being snap frozen in a bath of dry ice and ethanol and stored at -80^{o}C.

2.2.5 Retroviral transduction

Below is a general protocol followed for retroviral transduction. Modifications that were made depending on cell type are noted at the end. Pre-coated Retronectin plates were aspirated to remove the Retronectin before 250µl of retroviral supernatant was used to pre-coat the wells during cell harvest. This

retroviral supernatant was removed immediately prior to addition of suspension cells to be transduced.

2.2.5.1 Retroviral transduction of suspension cells

Cells were harvested and re-suspended at $6x10^5$ cells/ml. 500µl of this cell suspension was added to each well of the Retronectin plate, along with 1.5-2ml of retroviral supernatant. Plates were spun for 40minutes at 1000G and returned to the incubator at 37ºC, 5% CO_2 for two days before downstream processing.

2.2.5.2 Retroviral transduction of adherent cells

293T cells were transduced as follows: Cells were plated at $1x10^6$ cells per 6-well plate, with 2ml of medium plated in each well. The following day, plates were observed for confluence with the well selected for transduction based on confluence, growth and attachment profiles. Transduction was performed by removal of all media. This was replaced with 2ml of retroviral supernatant along with 20µg of polybrene. Cells were then returned to the 37ºC, 5% CO_2 incubator overnight. The above transduction process was repeated daily until the 293T cells achieved confluence, when they were sub-cultured into a $175cm^2$ flask. Cells were then processed downstream to determine transgene expression, and if appropriate, aliquots were cryopreserved and added to the group cell line bank.

2.2.5.3 Retroviral transduction of human PBMCs

Primary human PBMCs were isolated and stimulated as described in 2.2.3.5 Isolation of PBMCs. PBMCs were then harvested, counted and re-suspended at a concentration of $6x10^5$ cells/ml. The PBMC suspension was

supplemented with 400 I.U./ml of recombinant human IL-2 and 500µl of this cell suspension was transferred to each well of the retronectin coated plate. 1.5ml of retroviral supernatant was added to each well and cells were spun at 1000G for 40minutes at 37^{o}C prior to return to the 37^{o}C, 5% CO_2 incubator for two days before downstream processing.

2.2.6 FACS analysis

2.2.6.1 Assessing transduction efficiency by FACS

FACS analysis was performed to determine transduction efficiency. Transduced cells were harvested from retronectin-coated plates and either left to recover for 48-72hours or analysed straightaway, depending on the experimental requirement. If the construct used had a fluorescent marker gene included, cells were washed in 1x PBS buffer before being analysed on a CyAn (Dako), LSRFortessa or FACSVerse (both BD) flow cytometer. If the construct used had the RQR8 sort-suicide gene included, then transduction efficiency was analysed by CD34 expression, and therefore a relevant anti-CD34 (QBend10) antibody was used, if necessary performing a two-step staining process. Cells were washed in-between staining steps with 1x PBS and analysed on the flow cytometers described above. In order to obtain target cell populations, gating was performed on the acquired cells to obtain a live population, based on forward scatter (FSC) and side scatter (SSC), followed by gating on combinations of marker genes and/or fluorescently-stained cell surface markers as required.

2.2.6.2 General antibody staining protocol

In order to analyse cell surface expression of various proteins, FACS analysis was used. Cells to be analysed were harvested into 5ml FACS tubes. Tubes were filled with 1x PBS and spun at 400G for 5 minutes. Supernatant was discarded, the FACS tubes were blotted on paper towel and the cell pellet was

re-suspended by vortexing. The relevant volume of antibody was added as per the manufacturer's instructions and cells were vortexed briefly before being stored at room temperature in the dark for 15-30minutes. After staining, cells were again washed with 1x PBS and spun at 400G for 5minutes. Supernatant was discarded and the FACS tubes were blotted onto paper towel. Cells were then re-suspended in up to 500µl of 1x PBS and vortexed before being analysed on a flow cytometer. If multiple antibody staining steps were required, the staining cycle was repeated for each staining required, with a 1x PBS wash and spin performed between each step to remove any excess unbound antibody. FACS tubes were placed in wet ice pending flow cytometry analysis. Relevant non-transduced/non-stained/single-stained controls were included for benchmark comparison.

2.2.6.3 PFA fixation protocol

Where sample analysis was delayed overnight, following the final wash after antibody staining, samples were fixed by final re-suspension into 0.4% PFA-PBS solution and stored at 4^{o}C before flow cytometry analysis.

2.2.6.4 Intracellular staining

For intracellular IFNγ staining, the BD Cytofix/Cytoperm^{TM} kit was used. Firstly, GolgiStop^{TM} was incubated with the cells to be stained according to the manufacturer's instructions to stop and further secretion of intracellular cytokines. After GolgiStop^{TM} had been incubating with the cells for no longer than 12hours, cells were harvested and spun down at 400G for 5minutes. Cells were washed in 1x PBS as previously described, then stained with any relevant cell surface antibodies that were being included in the analysis. After staining for cell surface molecules, cells were washed in 1x PBS, then they were fixed and permeabilised using the solutions provided in the BD Cytofix/Cytoperm^{TM} kit, for 20minutes at 4^{o}C. Cells were then washed twice in

the BD Perm/Wash™ buffer and centrifuged as before. Cells were re-suspended in 50µl of BD Perm/Wash buffer and intracellular anti-IFNγ-FITC (eBioscience) antibody was added and incubated with cells at 4°C for 15minutes. After intracellular staining, cells were washed twice in 1x PBS before being analysed by flow cytometry.

2.2.6.5 Staining cells with secreted soluble antibody

Secreted versions of some antibody transgenes were cloned into the SFG expression vector. The secreted versions contain a human Fc region, which can be targeted using an anti-human Fc antibody. By using secreted versions of antibodies, it is possible to determine whether they bind to their reported target of interest or not. These constructs are transfected into 293T cells as described in 2.2.4.2 Transient transfection for expression testing. After 48hours, crude supernatant is removed and used to stain cells of interest. After cells of interest have been harvested into 5ml FACS tubes, up to 4ml of crude supernatant is added. Tubes are vortexed and incubated at room temperature for 15minutes in the dark. After 15minutes, cells are centrifuged at 400G for 5mins and supernatant is discarded. Cells are then re-suspended in the remaining fluid and 2.5µl of the relevant anti-human-Fc-Dylight antibody is added. Cells are vortexed again and incubated for a further 15minutes at room temperature in the dark before being washed in 1x PBS. Cells are then treated as they would be for a regular flow cytometry analysis. Successful acquisition of the fluorophore in the analysis indicates that the secreted antibody is successfully binding to its target on the cell surface.

2.2.6.6 FACS sorting of transduced cells

Cells needing to be sorted to obtain a pure population were harvested and stained (if required) as described in section 2.2.6.2 General antibody staining protocol, with the exception of the process being carried out in sterile conditions

with capped FACS tubes. After cell preparation, cells were re-suspended in 1-2ml of complete media. The machine used for FACS sorting was the MOFLO XDP (Beckman Coulter). Initially, a few cells were acquired and the relevant parameters were set on the software (Summit) to allow for sorting of the desired population. When parameters had been set, cells were run through the MOFLO XDP, and sorted cells were collected in a fresh FACS tube containing 1ml of FCS with 1mg/ml of antibiotic Normocin (Invivogen). After sorting, cells were removed from the MOFLO XDP, centrifuged and recovered into a $25cm^2$ or $75cm^2$ flask, also containing 1mg/ml Normocin. Cells were allowed to recover for 3-14 days and were regularly checked for any bacterial contamination. Once recovered, the cells were analysed by flow cytometry to verify their purity.

2.2.7 In vitro magnetic cell selections

When using the RQR8 sort-suicide gene as part of a multiple-construct expression vector, it was possible to perform cell selections using the MACS system (Miltenyi). This system was also used for $TCR^{+/-}$, $CD34^{+/-}$ and $CD56^{+/-}$ selections.

2.2.7.1 General magnetic bead selection protocol

Magnetic bead selections were performed on both immortalised cell line and primary human T-cells when required. For cell lines, 20µl of beads were used to select $<1.0x10^7$ cells, separated using an MS column. For human primary T-cells, 100µl of beads were used to select $<1.0x10^8$ cells, separated using LS columns. Beads were added to cell suspensions and incubated for 15minutes at 4^oC in the dark. Cells were then spun down at 400G for 5minutes and the supernatant was aspirated. Cells were re-suspended in MACS buffer before being run on the relevant column. For a positive selection, cells were added to LS or MS columns, run-through was discarded and bound cells were eluted

by addition of MACS buffer and using a plunger to wash off the bound fraction. Negative selections were performed using LD columns, with the unbound fraction kept and the bound fraction discarded. The required cells were then spun down at 400G for 5minutes. Supernatant was removed and cells were re-suspended in the relevant complete media. A cell count was performed and cells were cultured at appropriate densities as previously described.

2.2.7.2 T cell magnetic bead selection

As anti-TCR MACS beads were unavailable, a two-step protocol was used, where T cells were stained with aTCR-biotin antibody using the general antibody staining protocol (2.2.6.2 General antibody staining protocol). After washing in 1x PBS, cells were re-suspended in MACS buffer and streptavidin beads were used to bind to the biotin molecule on the aTCR antibody. Cells were then selected as described above on the relevant column.

2.2.7.3 NK cell magnetic bead selection

To exclude residual NK cells and potential lymphokine-activated killer cells prior to ^{3}H assays, samples were depleted of CD56-expressing effector cells. For negative depletions, 20µl of beads were used to select $<1.0x10^{7}$ cells, separated using LD columns. Where required, multiple separations were performed in tandem.

2.2.8 Cell-based assays

Several cell-based assays were performed to determine cell function, expression and interaction. These are described below:

2.2.8.1 T cell incubation assays

In order to determine the mechanism of apparent TCR knockdown achieved by the ER retention strategy (5.4.3 Knockdown *of TCR by aTCR-SEKDEL constructs in donor PBMCs*transduced and non-transduced autologous T cells were co-cultured together in various ways. Firstly, both sets of T cells were incubated together in a tissue culture-treated 24well plate at a 1:1 ratio for two days, followed by flow cytometry analysis as described in 2.2.6 *FACS analysis*. A repeat of this experiment using the same set-up was performed using a 6.5mm 0.4µM pore transwell (Corning) insert in several wells of a 24well tissue culture-treated plate to separate the non-transduced and the transduced T cell populations. After two days of co-culture, non-transduced T cells were removed and stained as previously described, followed by flow cytometry analysis.

2.2.8.2 ^{3}H-thymidine uptake proliferation assay

To perform this functional assay, 10^5 effector cells were plated in triplicate in U-bottomed 96 well plates with various ratios (16:1 to 1:1) of allogeneic irradiated transduced cells. For T cell irradiation, 30Gray of X-ray was used; for cell lines, 120Gray of X-ray was used. Irradiations were all performed using an AGO HS MP-1 X-ray irradiator. After 5 days of co-culture at 37ºC and 5% CO_2, wells were pulsed with 1µl (37Bq) ^{3}H-thymidine and incubated at 37ºC, 5% CO_2 for a further 20 hours. Cells were then cells were harvested to a filter mat using a Tomtec MachIIIM Harvester 96. MeltiLex wax scintillation was applied, and thymidine incorporation measured using a Wallac 1450 MicroBeta trilux beta-counter (Perkin Elmer). Specific proliferation was calculated by subtracting mean counts per minute (cpm) of effector and target alone control wells from those containing effector cells stimulated with target cells.

2.2.8.3 ^{51}Chromium release assay

Functional demonstration of the effector capability of donor PBMCs and NK cells was measured by chromium release assays performed against K562 cells expressing HLA molecules.

Chromium release was calculated as follows:

Chromium release = [(Experimental release – background release)*100] / (Maximum release – background release)

K562 cells were loaded with ^{51}Chromium as follows: approximately 1x10^{6} target cells were isolated for each target. Cells were harvested by centrifugation, supernatant was discarded and the residual cell pellet was gently re-suspended. 3.7MBq of ^{51}Cr labelled sodium chromate (Perkin Elmer) was added to each tube of targets. Cells were incubated for 1hour and gently agitated every 15minutes. Following loading, residual chromium was removed by five consecutive complete media washes, before the cells were re-suspended in media ready for use in the assay.

The K562 cell line was transduced with either the SFG.HLA-A2.eGFP or SFG.HLA-G.eBFP2 construct. Following transduction, cells were purified by MOFLO cell sorting according to fluorescent gene expression. Cells were allowed to recover for 24hours before they were used in the assay. Target cells were then combined with PBMCs or NK cells (both derived from the same donor). Effector cells were then incubated with the relevant target cell line for 2hours before obtaining a readout using a gamma counter.

2.2.9 Statistical Analysis

Statistical analysis was performed where indicated, using either Graphpad Prism software or Microsoft Excel to calculate averages, means, standard

deviation (SD) or standard error of the mean (SEM). Relevant statistical analyses were performed on data where appropriate in order to gain insight into the significance of results. For data presented in column format, values were calculated using one-way ANOVA analyses.

Where indicated:

‘*’ - $p \leq 0.05$

‘**’ - $p \leq 0.01$

‘***’ - $p \leq 0.001$

‘****’ - $p \leq 0.0001$

Chapter Three: HLA Class I knockdown

3.0 Aims

- To investigate various strategies that could result in the knockdown of HLA Class I from a cell surface.
- To determine which strategy is the most successful in achieving HLA Class I knockdown.
- To analyse the functional effect of the HLA Class I knockdown.

3.1 Introduction

HLA is expressed on the surface of most human cells. Its major function is in immunity, as HLA molecules are the mechanism by which peptides are presented on the cell surface to either activate or prevent an immune response. The majority of HLA molecules come under the classical Class I (HLA-A, -B and -C) or non-classical Class II (HLA-E, -F and -G) categories. These are all transmembrane glycoproteins that comprise a heterodimer of a polymorphic 45-kilodalton (kDa) chain and a non-polymorphic 12kDa beta-2-microglobulin (β2m) chain (Bjorkman & Parham 1990; Shawar et al. 1994).

When cells are infected by virus, or are transformed into cancerous cells, HLA expression is often down-regulated in order to bypass the immune surveillance that would otherwise eliminate the unhealthy cells (Hicklin et al. 1999). From immunohistochemical analysis of surgically excised tumours, multiple HLA expression phenotypes have been noted, including complete HLA Class I down-regulation, selective loss of a HLA Class I haplotype, selective down-regulation of a HLA locus, selective loss or down-regulation of a HLA allele, and a combination of the above to create a complex phenotype (Ferrone & Marincola 1995; Garrido et al. 1997). Several of these phenotypes have been displayed in one tumour sample, demonstrating the ability of cancerous cells to modulate HLA Class I expression to ascertain the optimal method of preventing an immune response.

The molecular basis of these phenotypes is often due to mutations in key genes, with ‘hot spots’ of mutations localised to the β2m gene, frequently reported as being responsible for complete HLA Class I loss in colon carcinoma and melanoma cells (Bicknell et al. 1994; Hicklin et al. 1998). Other mutations modulate the ability of cells to process antigen and fold HLA molecules. Genes including latent membrane protein (LMP)-2, LMP7, TAP1 and TAP2 were found to be down-regulated in small cell lung carcinoma cell lines (Restifo et al. 1993). In a study investigating viral infection of cell lines by Human immunodeficiency virus (HIV), HLA Class I was shown to be down-regulated a few days post-infection, and that peripheral CD4+ T cells affected by down-regulated HLA Class I were targeted less by allogeneic Class I-restricted CTLs compared to uninfected cells (Kerkau et al. 1989).

3.2 Approaches to HLA Class I Knockdown

There are various ways in which proteins can be eradicated from a cell. They can be targeted at the genomic level by methods such as ZFNs (discussed in chapter five); at the mRNA level by siRNA or microRNA (miRNA); at protein level by retention sequences and antibodies (Pelham 1990; LGC 2012). As seen in the above section, transformed cells and viruses are able to modify the HLA Class I expression in various ways. The aim was to mimic some of these strategies to achieve HLA Class I knockdown in healthy, non-transformed cells.

To target mRNA, the most commonly used technology is siRNA, although miRNAs are becoming more popular as their mechanisms and targets are better elucidated. siRNA technology was discovered by Fire et al. (1991) and has subsequently been shown to be effective at knocking down mRNA in multiple model organisms. siRNA works by using small 20-25 nucleotide-length RNAs which assemble into RNA-induced silencing complexes (RISCs), which are then able to cleave the target mRNA, leading to its degradation (Whitehead et al. 2009). The advantage of using siRNA as a knockdown strategy is that the molecule is very small and can target a specific sequence

of mRNA. Conversely though, siRNA may be recognised as foreign and destroyed and it may cause off-target interactions. It is also difficult to get complete knockdown due to the siRNA machinery which can get saturated and the recent ability to elucidate the function of microRNAs may mean that these have become the optimal method of RNA interference (Boudreau et al. 2008; Meister et al. 2004).

In order to obtain knockdown of a protein, one strategy that was used in this project was to combine the specificity of an antibody against a protein with a retention sequence to retain the protein within the cell and prevent its surface expression. Retention sequences were first characterised by Munro and Pelham (LGC 2012), who showed that an amino acid sequence of 'KDEL' which was present on the C-terminus of three luminal ER proteins was responsible for their retention in the ER. Following on from this, it was shown that this sequence, when attached to various proteins, could be used to retain them in the ER and other locations (Pelham 1990; Andres et al. 1990). To target a protein in a specific manner and retain it within the ER, the strategy in this study required a molecule attached to a retention sequence that would bind to the protein of interest. This approach allows for a specific targeting of a protein and for intracellular retention, but the drawback is that it might cause ER stress due to the large amount of protein being retained in the ER. This could cause the activation of the unfolded protein response (UPR) and would lead to up-regulation of ER stress proteins including inositol-requiring enzyme (IRE)-1, activating-transcription factor (ATF)-6 and protein kinase RNA-like endoplasmic reticulum kinase (PERK), leading to ER-associated degradation (ERAD), translational attenuation and possible apoptosis (Rutkowski & Kaufman 2004; Schröder & Kaufman 2005). Knockdown of HLA Class I molecules by using a protein targeting method was preferable to using DNA editing molecules as it removed the risk of mutations caused by non-homologous end joining (NHEJ), making this project safer and more viable. It also allows the use of a viral packaging and delivery system and therefore can be combined with other strategies, reducing the number of selection stages to obtain a homogenous cell product.

3.3 Testing of constructs

Several siRNA and protein based strategies were investigated to cause knockdown of HLA Class I, with the aim of identifying the optimal strategy to take forward in the overall project. Some of these strategies have been described by other investigators, and some are novel. The approaches can be categorised as: (i) viral proteins (ii) dominant-negative β2-microglobulin (iii) siRNA (iv) single chain Golgi retention molecules generated from BBM1 and BB7 hybridomas (scFvs).

3.4 Viral proteins

It has been shown that the use of viral transgenes results in a decrease in the ability to present antigens on the cell surface MHC Class I molecules by disrupting the CD8+ CTL response (Radosevich et al. 2003). The viral proteins used were herpes simplex virus (HSV) protein ICP47 and human cytomegalovirus (HMCV) protein unique short glycoprotein (US)-11. ICP47 is a small 88 amino acid protein that binds to transporter associated with antigen processing (TAP). This prevents peptide translocation and loading onto MHC Class I molecules in the ER, leading to the degradation of immature MHC Class I. US11 is a 215 amino acid protein which ubiquitinates MHC Class I molecules in the ER, leading to retrotranslocation to the cytosol and subsequent degradation by the proteasome. Both of these proteins were cloned using oligonucleotide assembly (**Figure 3**). After initial testing, both were shown to have promising effects, which prompted the step of cloning both proteins together in one construct, joined by a 2A peptide. In a similar study by Berger et al. (2000), ICP47 and US11 were both tested to determine their efficacy at preventing transgene expression by $CD8^+$ T cells. The aim here was to attempt something similar but to determine whether the use of one or both viral proteins was sufficient to prevent a response, and also, when used with the sort-suicide gene RQR8, to demonstrate the ability to generate

a homogenous pool of modified cells instead of doing this by FACS as in the study above.

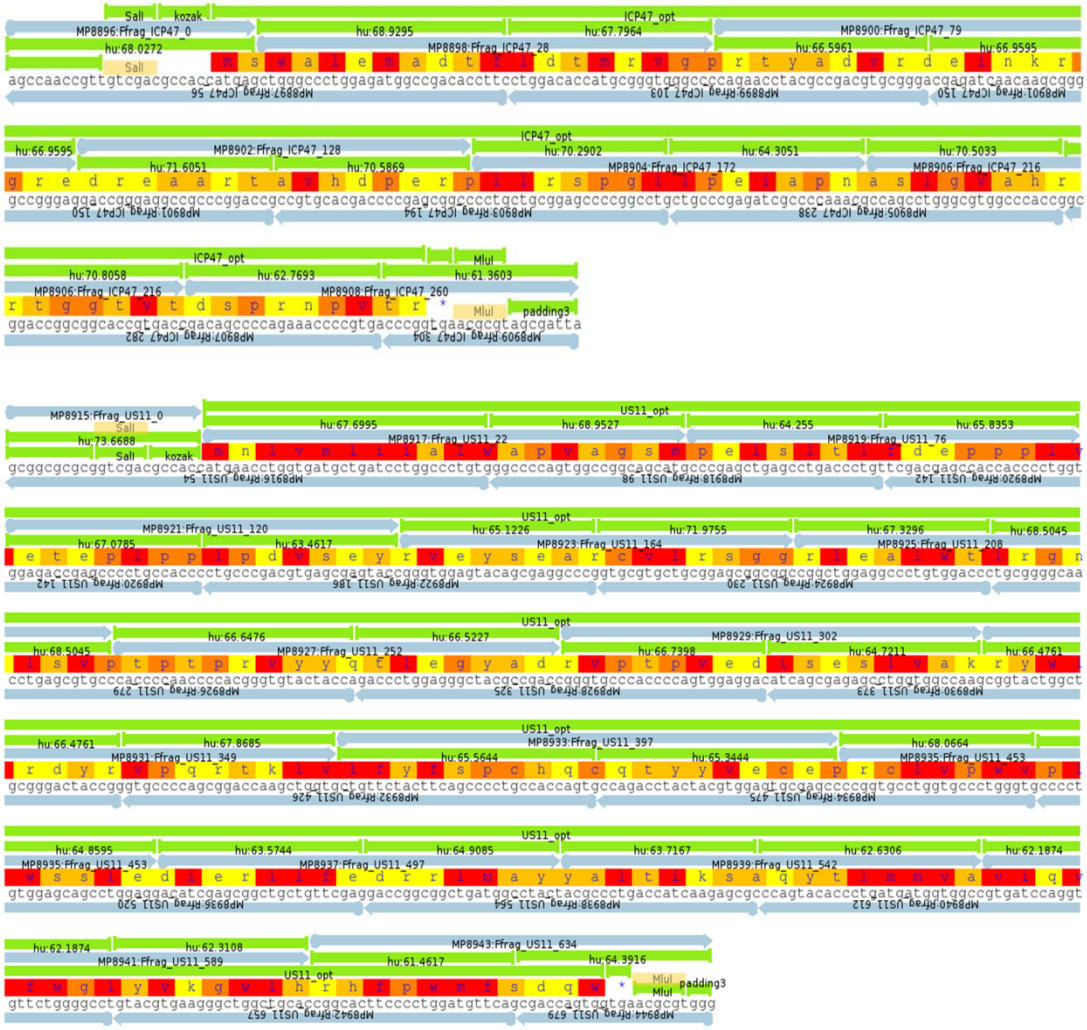


Figure 3 – Construct designs for the viral proteins ICP47 (top) and US11 (bottom). Blue arrows indicate oligonucleotides used to build genes through oligonucleotide assembly, multi-coloured blocks denote amino acid sequence.

3.5 Beta-2-microglobulin

β-2-microglobulin (β2m) is a subunit of the MHC Class I molecule that has been shown to function as a chaperone for MHC Class I molecule folding (Fink 1999) and is also a major subunit of the molecule itself (Bix & Raulet 1992; Seong et al. 1988; Zijlstra et al. 1990). A mutant β2m defective in binding to the α1 and α2 subunits of the MHC Class I molecule has been shown to block

the extracellular folding of MHC Class I molecules, thereby knocking down HLA from the cell surface (Hill et al. 2003). These β2m mutants were generated by using oligonucleotide assembly to create mutant forms of wild-type β2m (**Figure 4**). We generated the same mutants as Hill et al. (2003), namely β2m with a D53K mutation, β2m with a W60A mutation and β2m with both the above mutants (D53K/W60A).

Figure 4 – Construct designs for mutant β-2-microglobulin molecules. From top to bottom, wild-type, D53K, W60A and D53K/W60A mutant designs. Blue arrows indicate oligonucleotide primers to amplify β2-microglobulin from the EST_5502428 clone; multi-coloured blocks denote amino acid sequence.

3.6 siRNA

siRNA is a powerful tool that has been developed to knockdown specific targets. Vector strategies have been developed which allow continuous

delivery of siRNA from an integrated vector. This is another strategy that was used to cause HLA Class I knockdown by using two siRNAs targeting β2m, as previously described (Gonzalez et al. 2005; Haga et al. 2006). We also generated our own designs against β2m (Thermo Scientific 2011) (**Figure 5**). These were generated by single-pair oligonucleotide annealing and assembly before being cloned into the pSuperRetro.eGFP plasmid, which drives the production of siRNA using the RNA polymerase III promoter.

1. GATCCCCGGAGATCACACTGACCTGGCATTTGTGTAGTGCCAGGTCAGTGTGATCTCCTTTTTAAGCT
2. GATCCCCGCTGTGGTGGTGCCTTCTGGTTCAAGAGACCAGAAGGCACCACCACAGCTTTTTAAGCT
3. GATCCCCCTCCAAAGATTCAGGTTTATTCAAGAGATAAACCTGAATCTTTGGAGTTTTTAAGCT
4. GATCCCCCAGAGAATGGAAAGTCAAATTCAAGAGATTTGACTTTCCATTCTCTGTTTTTAAGCT
5. GATCCCCGCAGAGAATGGAAAGTCAATTCAAGAGATTGACTTTCCATTCTCTGCTTTTTAAGCT
6. GATCCCCCCAAGATAGTTAAGTGGGATTCAAGAGATCCCACTTAACTATCTTGGTTTTTAAGCT
7. GATCCCCGTGTGAACCATGTGACTTTTTCAAGAGAAAAGTCACATGGTTCACACTTTTTAAGCT

Figure 5 – Sequences of siRNA used to target knockdown of β-2-microglobulin. Blue highlighted sequence shows sense and anti-sense DNA, green highlighted texts shows hairpin loop sequence. 1 – 'Outside_01'; 2 – 'Outside_02'; 3 – 'Own_01'; 4 – 'Own_02'; 5 – 'Own_03'; 6 – 'Own_04'; 7 – 'Own_05'. Sequences 3-7 were designed using siDesign-Center online software.

3.7 scFvs derived from hybridomas

The BBM1 hybridoma was obtained from ATCC (LGC 2012) and heavy and light chains were extracted and cloned together to create an scFv against β2m, using the Dubel primers (Toleikis et al. 2004) (**Figure 6**). A paper by Mhashilkar et al. (2002) showed knockdown of HLA expression using intrabodies derived from the BB7 hybridoma. Intrabodies are antibodies expressed in various intracellular compartments that are designed to modify or block target molecule function or expression. They work by either neutralising the target protein through direct binding to the functional domain, interfering with binding partners or by diversion of the target to a different subcellular compartment to prevent function (Cardinale & Biocca 2010). In the study by Mhashilkar et al.

(2002), two scFvs against β2m were created that would knockdown expression of human MHC Class I molecules by retaining MHC Class I in the ER (**Figure 7**). This was achieved by linking the scFv to a Golgi retention signal. Complete cellular surface knockdown was achieved in Jurkat T cells stably expressing an ER-directed sFvhMHCI intrabody. This was then used in multiple human cell lines with down-regulation of MHC Class I expression, even under inflammatory conditions. This shows that it is possible to achieve a reduced level of MHC Class I expression on the cellular surface. The two scFvs from this study and one extracted from the BBM1 hybridoma were cloned into the SFG vector containing a marker gene.

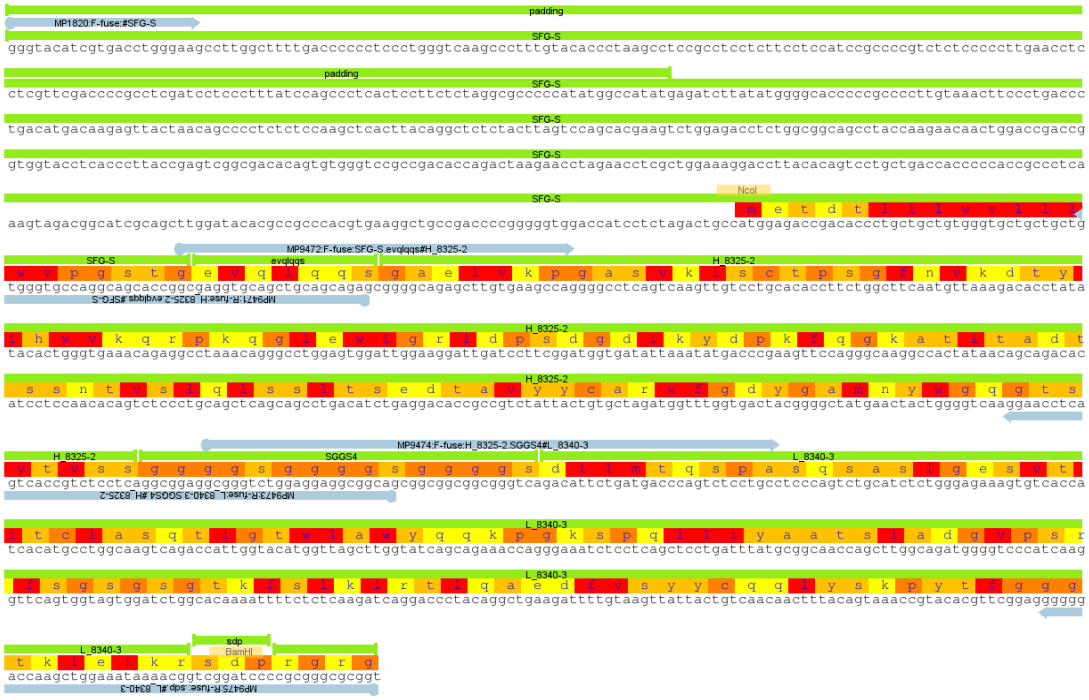


Figure 6 – Construct design for scFv extracted from the BBM1 hybridoma. Blue arrows indicate oligonucleotide primers used to amplify fragments of heavy and light chains from products extracted from the hybridoma using Dubel primers. Multi-coloured blocks indicate amino acid sequence.

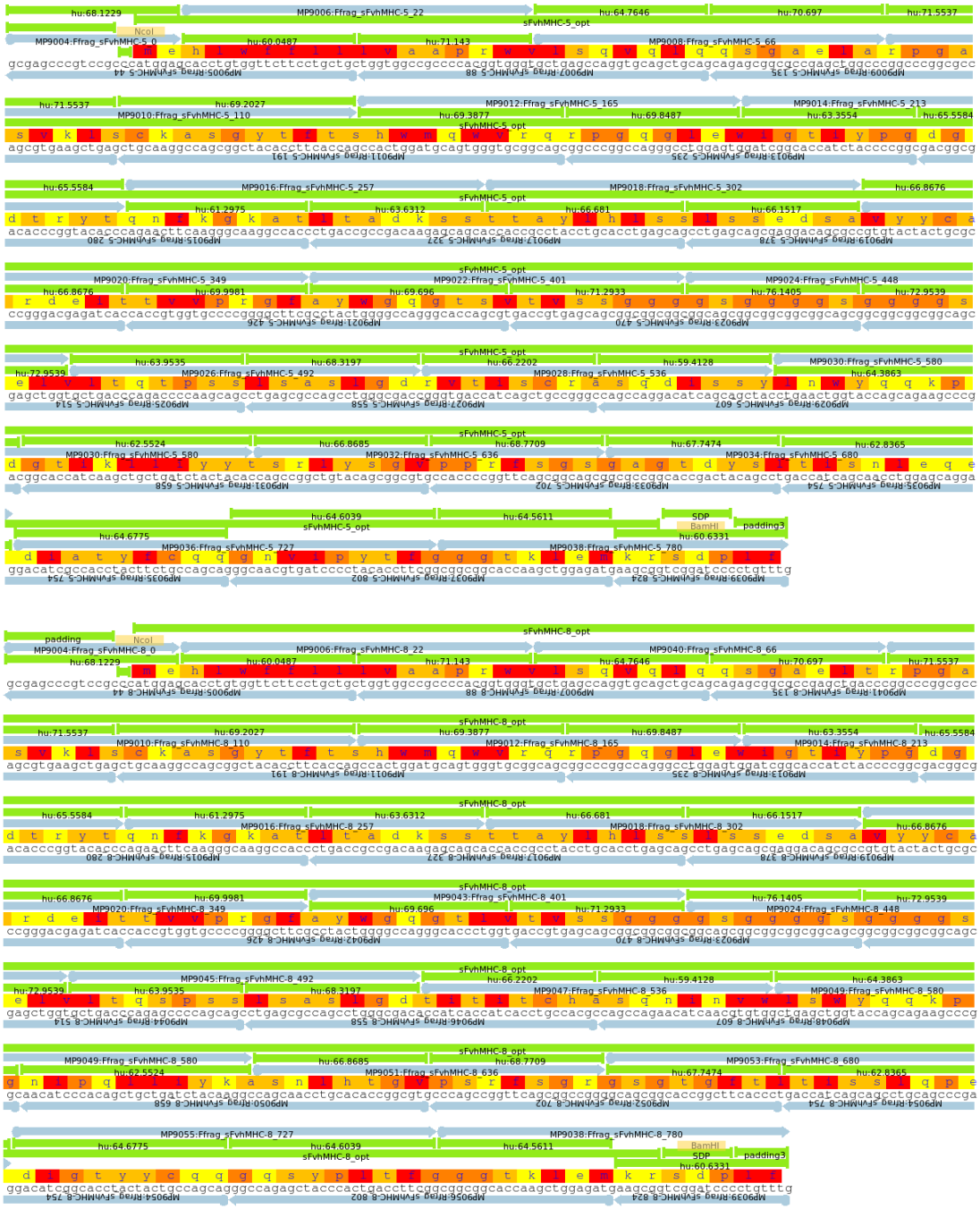


Figure 7 – Construct design for oligonucleotide assembly of scFvs derived from Mhashilkar et al. 2002. sFvhMHC-5 (top design); sFvhMHC-8 (bottom design). Blue arrows indicate oligonucleotides; multi-coloured blocks indicate amino acid sequence.

3.8 Transduction of constructs in cell lines

After assembly of the various constructs, retroviral supernatant (RD114) was produced and each of the constructs was individually transduced into the SupT1 cell line to assess the efficacy of HLA Class I knockdown. Knockdown was assessed by HLA Class I cell surface expression, measured by anti-HLA-A/B/C antibody staining (**Figure 8** and **Figure 9**). The anti-HLA-A/B/C antibody is a mAb derived from the hybridoma clone G46-2.6 and binds to a monomorphic epitope on the alpha chain of the MHC Class I antigen (Barclay et al. 1997; BD Pharmingen 2011).

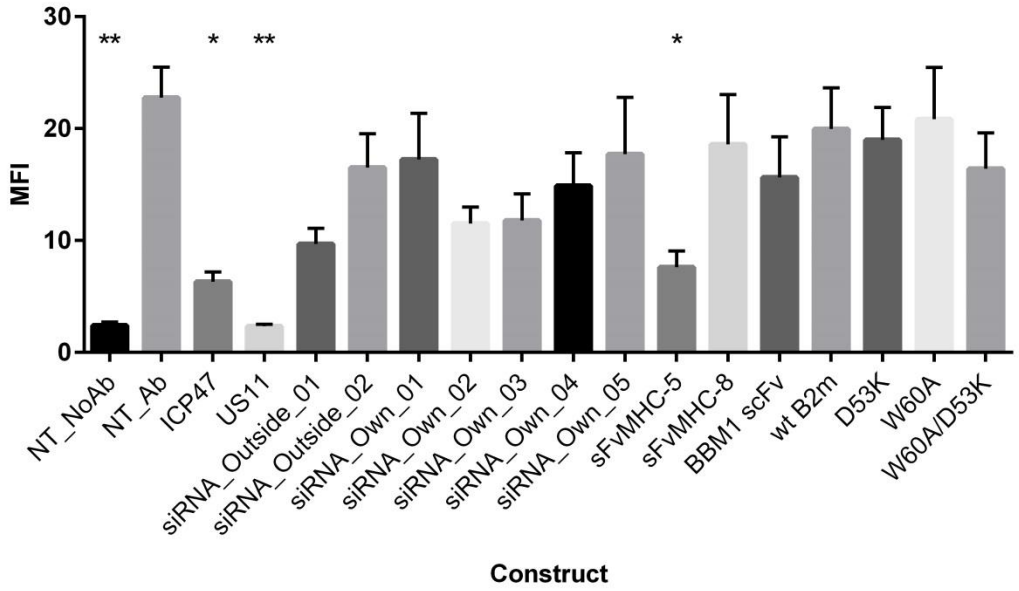


Figure 8 – HLA knockdown in SupT1 cell line as measured by mean fluorescence intensity (MFI). Surface expression was measured using anti-HLA-A/B/C-APC (BD) (not titrated). Error bars show S.E.M. (NT – non-transduced; NoAb – No antibody used; Ab – antibody used). Experiment repeated three times. One-way ANOVA analysis performed to calculate significance.

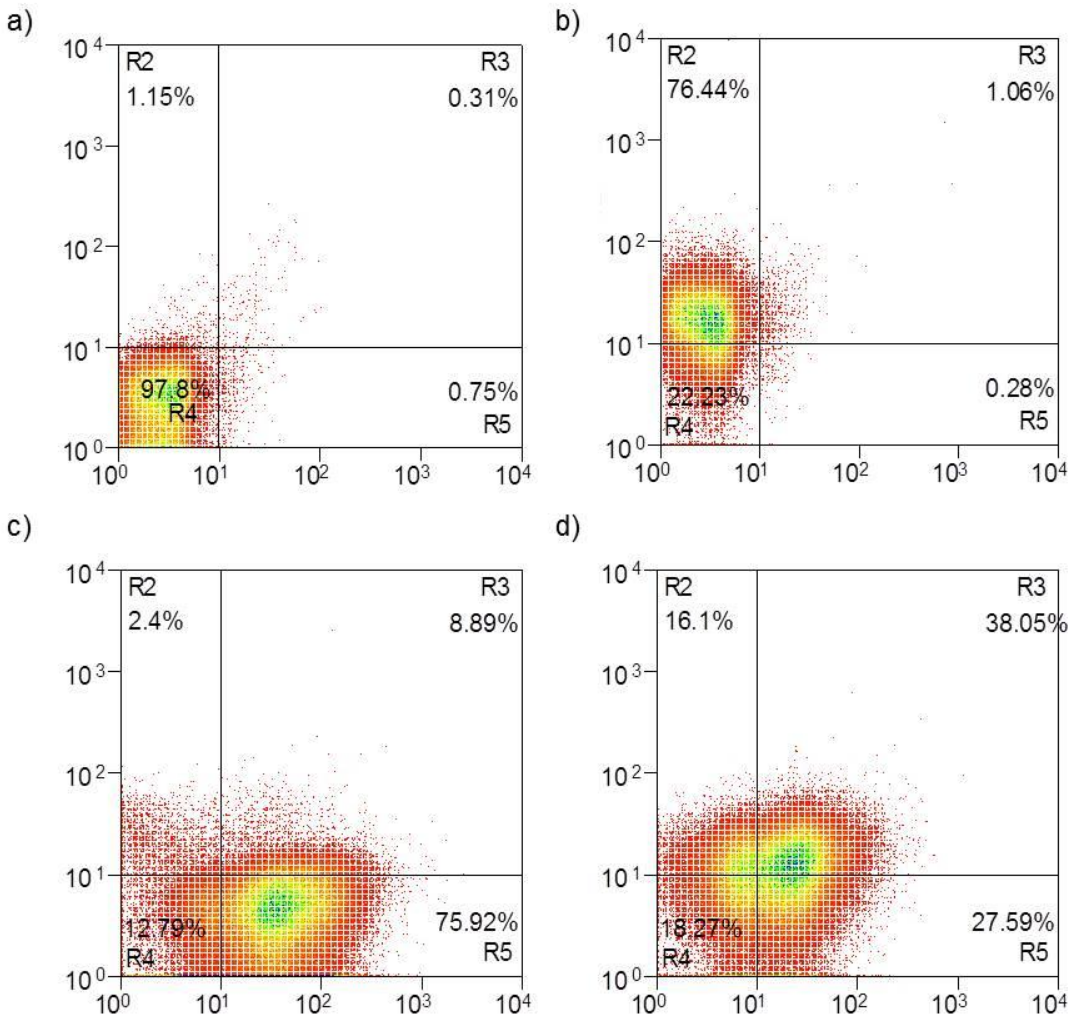


Figure 9 – FACS plots for HLA Class I knockdown in SupT1 cells (same as in **Figure 8**). Axes show: x-axis – eBFP2 marker gene; y-axis – aHLA-A/B/C/APC antibody (BD). a) Unstained cells b) NT stained cells c) Cells transduced with ICP47 construct d) Cells transduced with WT β2m construct. NT – non-transduced.

From these results, it can be seen that the two viral proteins, ICP47 and US11, are the two most effective constructs at achieving HLA Class I knockdown. Both viral proteins act by targeting the HLA Class I molecule as opposed to targeting β2m, therefore it appears that this is the more effective method of disrupting HLA Class I expression.

3.9 Expression of HLA in K562 cell line

3.9.1 Initial K562 cell line modification

In order to further verify the knockdown achieved by the viral proteins, a HLA negative cell line was needed to act as a negative control. The K562 cell line is of an erythroleukaemia type and lacks expression of the necessary MHC molecules to prevent NK cell activation (Britten et al. 2002) (**Figure 10**).

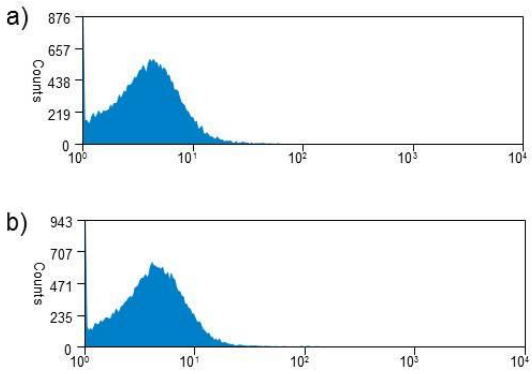


Figure 10 – Lack of HLA Class I expression in the K562 cell line. a) K562 cells with no antibody b) K562 cells with anti-HLA-A/B/C-APC antibody added. Repeated once.

The K562 cell line was modified with a construct containing HLA-A*0201 linked to β2m with a serine-glycine linker. The vector backbone was SFG. Retroviral supernatant was generated and used to transduce the K562 cells (**Figure 11**).

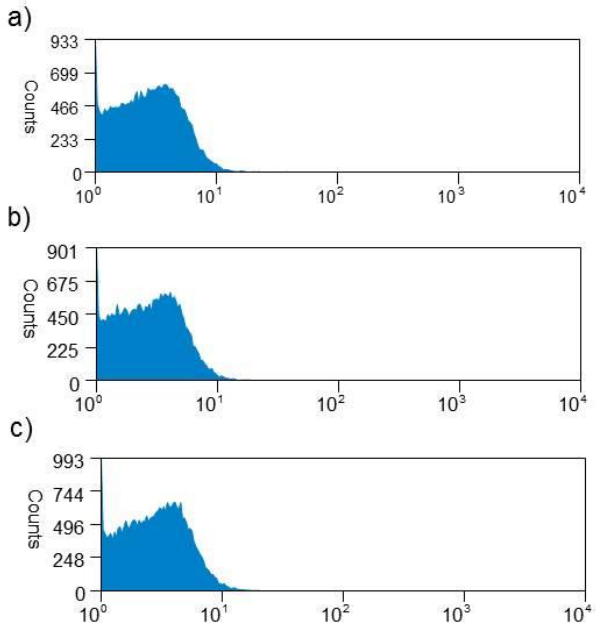


Figure 11 – HLA-A*0201 transduction in K562s. a) NT K562s without Ab b) NT K562s with anti-HLA-A/B/C-APC antibody added c) HLA-A*0201 transduced K562s with anti-HLA-A/B/C-APC antibody added. (NT – non-transduced). Repeated once.

This gene transfer seemed to have failed, leading to the conclusion that there may be something wrong with the HLA-A*0201 gene, and therefore the vector was sequenced. After sequencing, a non-conservative mutation was discovered, resulting in a serine to proline mutation (**Figure 12**). From searching the Immunogenetics (IMGT) database, this is not a recognised single-nucleotide polymorphism (SNP) or other polymorphism and was therefore determined to be the likely cause of the failure in HLA-A*0201 expression in the K562 cell line.

Align.	DB:ID	Source	Length	Score	Identities	Positives	E()
1	IMGTHLA:HLA00005	A*02:01:01:01 1098 bp	1098	1821	99.0	99.0	4.8E-189

```
>IMGTHLA:HLA00005 A*02:01:01:01 1098 bp
        Length = 1098

 Score = 1821 (646.1 bits), Expect = 4.8e-189, P = 4.8e-189, Group = 1
 Identities = 338/339 (99%), Positives = 338/339 (99%), Frame = +1

Query:      1 HSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDG 60
              HSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDG
Sbjct:     79 HSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDG 258

Query:     61 ETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKD 120
              ETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKD
Sbjct:    259 ETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKD 438

Query:    121 YIALKEDLRPWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRT 180
              YIALKEDLR WTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRT
Sbjct:    439 YIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRT 618

Query:    181 DAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQ 240
              DAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQ
Sbjct:    619 DAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQ 798

Query:    241 KWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEPSSQPTIPIVGIIAGLVLFGAVITGAV 300
              KWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEPSSQPTIPIVGIIAGLVLFGAVITGAV
Sbjct:    799 KWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEPSSQPTIPIVGIIAGLVLFGAVITGAV 978

Query:    301 VAAVMWRRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV 339
              VAAVMWRRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV
Sbjct:    979 VAAVMWRRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV 1095
```

Figure 12 – Alignment of original HLA-A*0201 construct (top line of sequence) with reference sequence from IMGT database (bottom line of sequence). Serine to proline mutation shown in red.

*3.9.2 Repair of β2m-L-HLA-A*0201 construct*

In order to repair the mutation in the HLA-A*0201 gene, oligonucleotide primers were designed by Dr Pule (**Figure 13**). In a PCR, the 5’ forward external primer and the internal reverse primer were used to amplify the first fragment using the original construct as the template. The internal forward primer and the 3’ reverse external primer were used to amplify the second fragment, again using the original construct as a template. These two ‘half’ fragments were fused together in the second reaction using the primers at the 5’ and 3’ ends of the design (below).

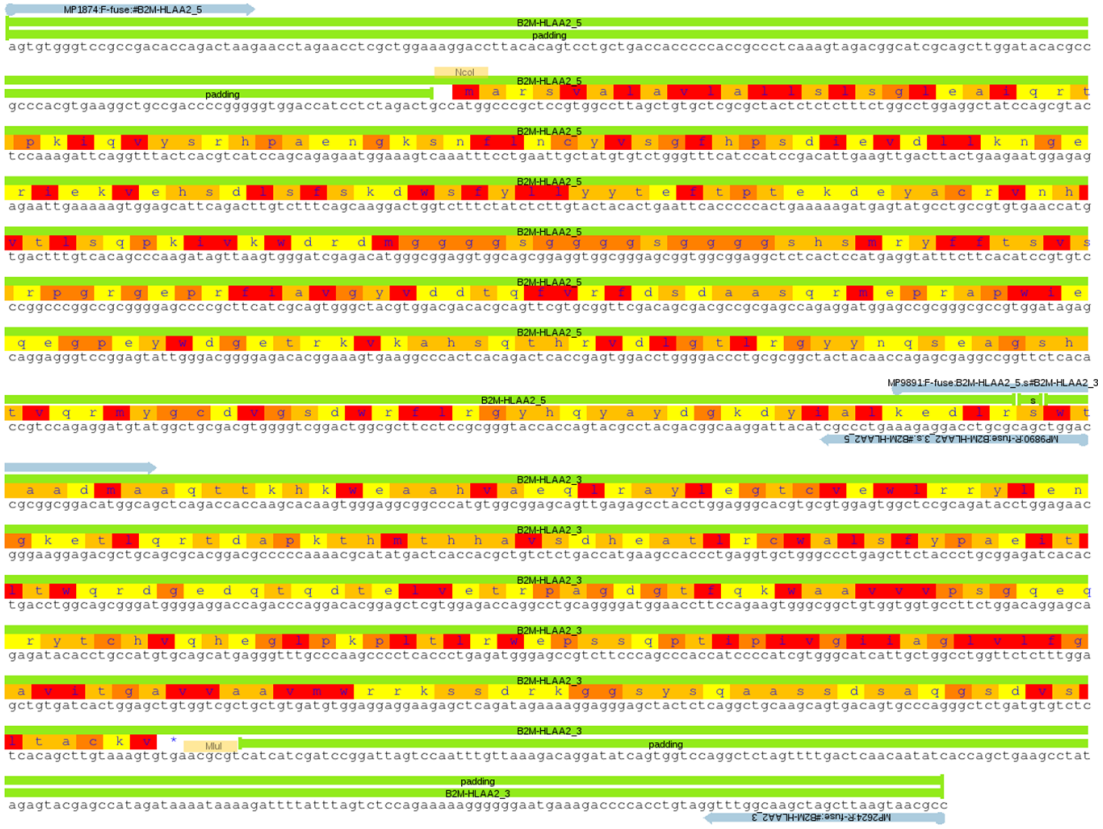


Figure 13 – Design for repair of β2m-L-HLA-A*0201 construct. Blue arrows show oligonucleotides used (and primer orientation), beige boxes show restriction enzyme sites, red/orange/yellow boxes show amino acid sequence, plain text shows DNA sequence.

It was also decided to create a construct that expressed HLA-A*0201 on its own. In order to do this, the β2m gene needed to be removed from the original β2m-L-HLA-A*0201 construct. This was again done using oligonucleotide primers designed by Dr Pule. This construct required three initial reactions before fusing the fragments together (**Figure 14**). A construct containing β2m on its own had previously been assembled, allowing for two strategies to be tested here – one of having β2m and HLA-A*0201 in one construct and the other of having the two molecules in separate constructs. Correct repair of HLA-A*0201 and removal of β2m was verified by DNA sequencing before the constructs were tested in K562 cells (**Figure 15**).

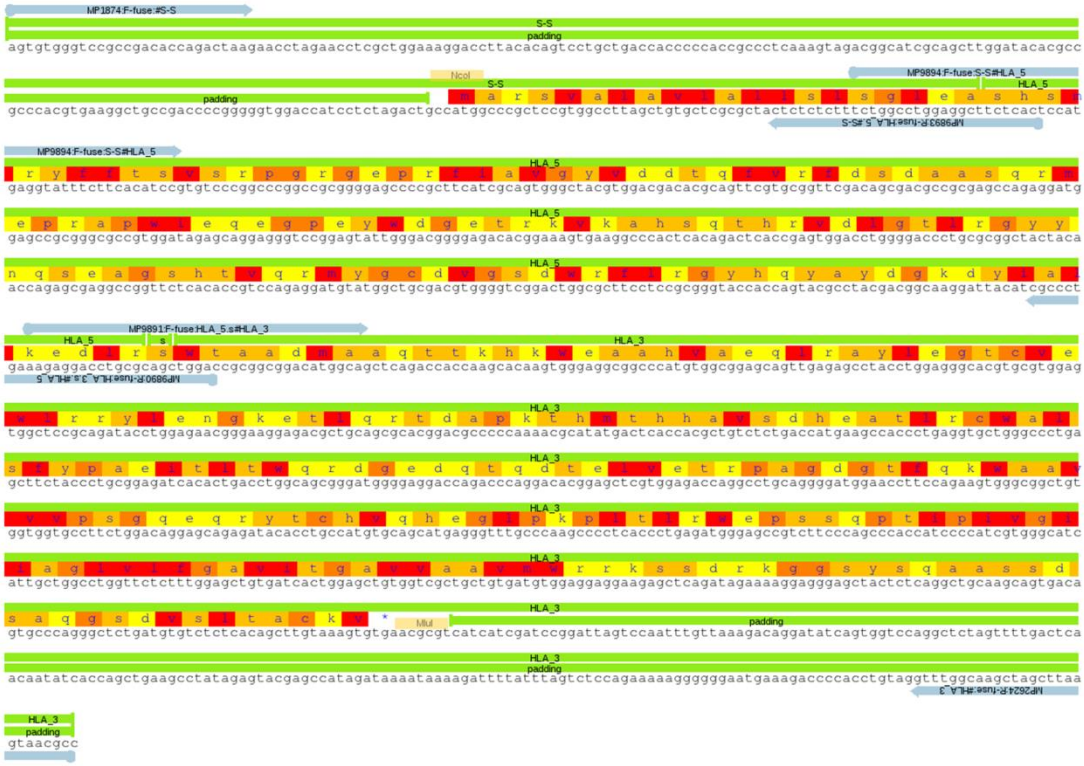
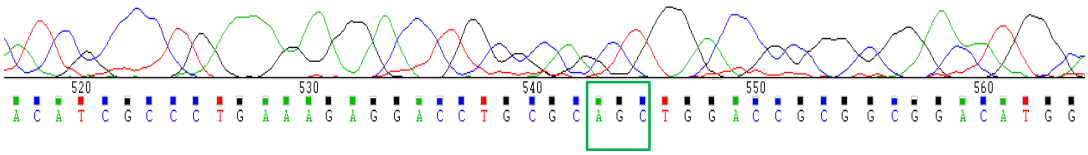


Figure 14 – Design for repair of HLA-A*0201 construct and removal of β2m. Blue arrows show oligonucleotides used (and primer orientation), beige boxes show restriction enzyme sites, red/orange/yellow boxes show amino acid sequence, plain text shows DNA sequence.

β2m-L-HLA-A*0201 Construct



HLA-A*0201 Construct

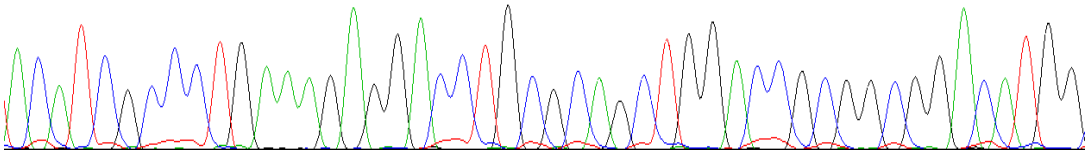


Figure 15 – Sequencing results from the repaired HLA-A*0201 constructs. Green boxes show codon for serine residue, correctly repaired from proline.

3.9.3 HLA Class I transduction in K562 and Daudi cell lines

These two constructs were then used to transduce the K562 cell line. As one construct did not have β2m included, a construct containing the β2m gene was also transduced into the K562 cell line. The transductions of HLA-A*0201 and β2m were done sequentially, not together. The construct containing both β2m and HLA-A*0201 failed to transduce successfully, but the doubly-transduced cells did express low levels of HLA-A*0201, with 6.22% of the cells being both eGFP and eBFP2 positive (**Figure 16**). Studies have shown that exogenous β2m has caused apoptosis in K562 cells and other cell lines (Wu et al. 2002; Gordon et al. 2003). As the doubly-transduced cells did express a low level of both molecules, this was not investigated further.

In order to purify the double positive population, cells were put through the MOFLO XDP sorter to allow selection of transduced cells, based on eBFP2 and eGFP co-expression (**Figure 17**). Cells were sorted to around 90% purity and maintained in culture for fourteen days. This was to allow them to recover and to ensure that no bacterial or microbial contamination had occurred during

the sorting process. Cells maintained the levels of HLA Class I expression, allowing for their use to test the HLA knockdown viral proteins.

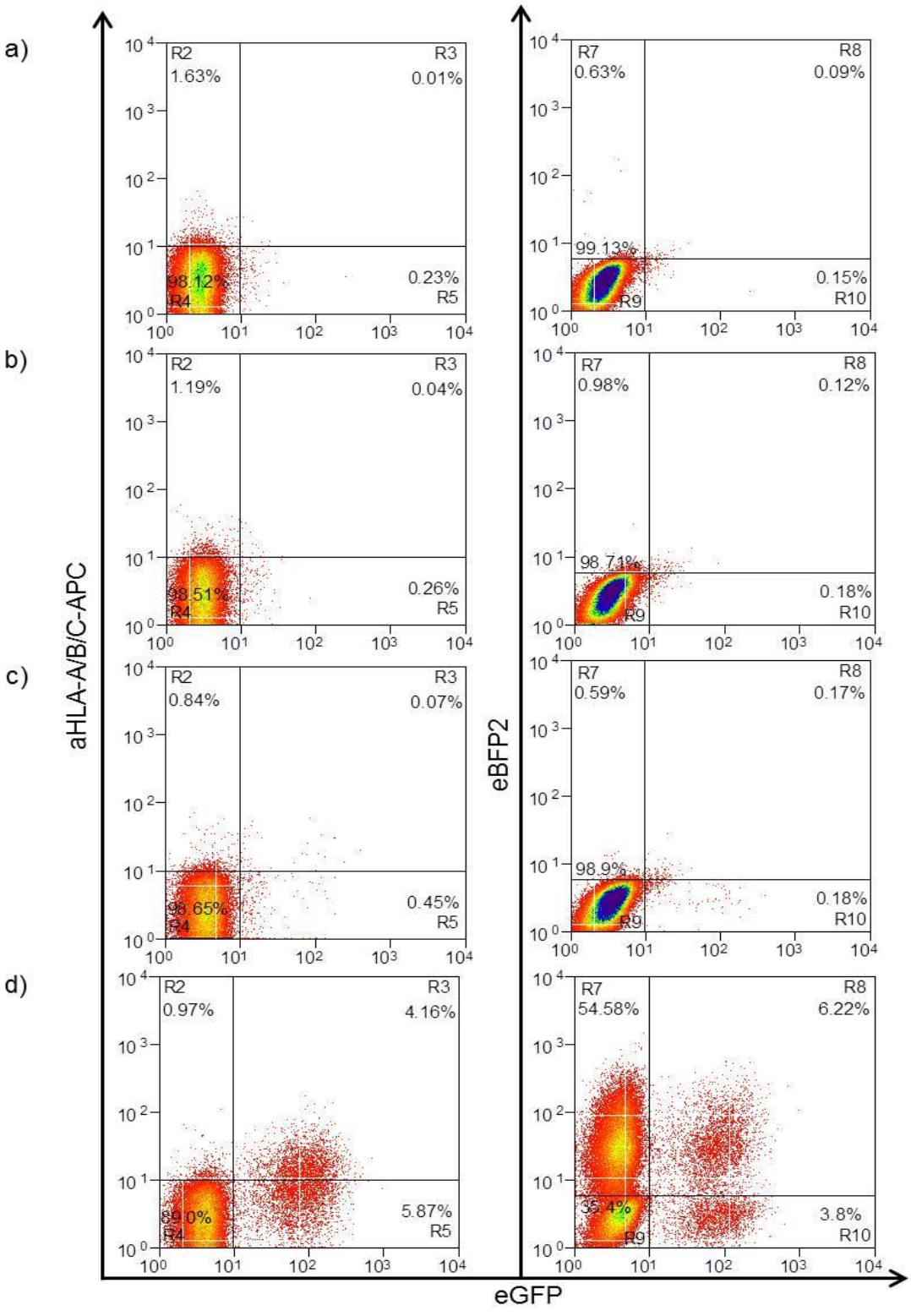


Figure 16 – Transduction of repaired HLA-A*0201 constructs into K562 cells. a) NT K562s with no antibody added b) NT K562 cells with anti-HLA-A/B/C-APC antibody added c) K562 cells transduced with β2m-L-HLA-A*0201 construct (eGFP marker gene) d) K562 cells transduced with separate HLA-A*0201 (eGFP) and β2m (eBFP2) constructs. Percentage shows proportion of double-positive cells. Left column shows HLA Class I expression, right column shows expression of marker genes. Repeated once.

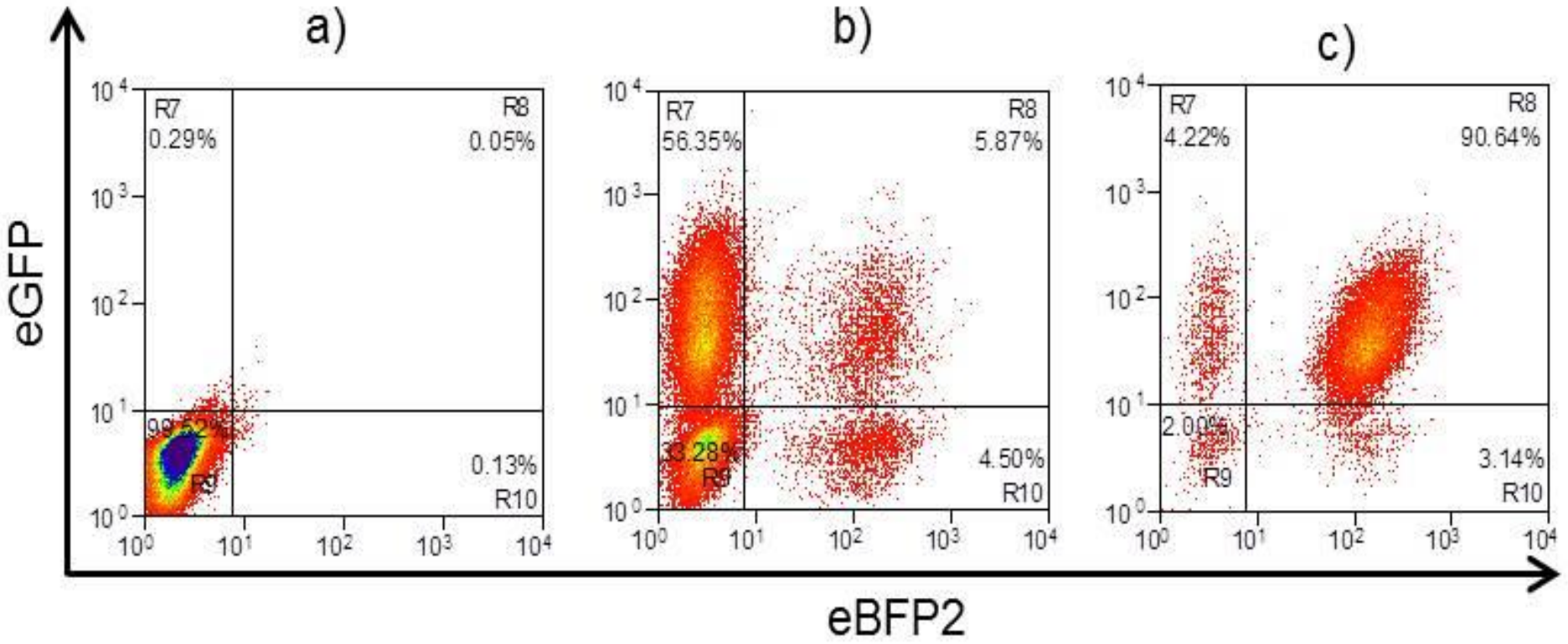


Figure 17 – FACS sorting of transduced K562s. a) NT K562s b) Unsorted K562 cells transduced with β2m construct (eBFP2 marker gene) and HLA-A*0201 construct (eGFP marker gene) c) FACS sorted K562 cells transduced with β2m construct (eBFP2 marker gene) and HLA-A*0201 construct (eGFP marker gene). Percentage shows proportion of cells in R3. Repeated once.

120

*3.9.4 Transduction of viral proteins into HLA-A*0201 expressing K562 cells*

After FACS sorting and allowing the cells to recover, the HLA-A*0201 positive K562 cells were transduced, using retrovirus (RD114-pseudotyped), with the viral proteins US11 and ICP47 (**Figure 18**). As can be seen, the effect of the ICP47 viral protein was very minimal, but the effect of US11 was

distinguishable. As the result here was not conclusive, it was decided to proceed with the two viral proteins by testing them in primary cells.

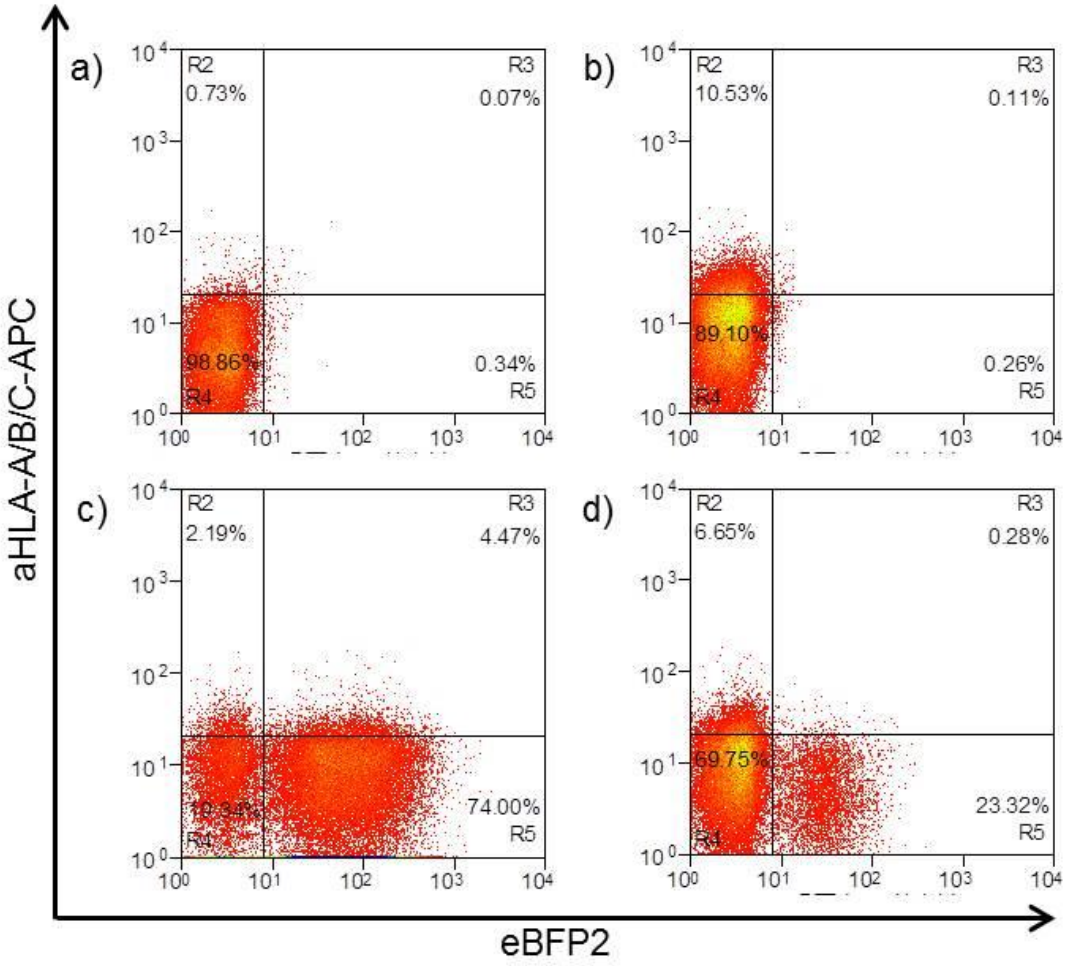


Figure 18 – Transduction of viral proteins into HLA-A*0201 K562 cell line. Top row: a) NT HLA-A2*0201 K562 cells with no antibody added b) NT HLA-A2*0201 K562 cells with aHLA-A/B/C-APC antibody added. Bottom row: HLA-A*0201 K562s transduced with c) ICP47 d) US11 (NT – non-transduced). Both constructs have eBFP2 marker gene; cells shown are eGFP-gated (HLA-A*0201 construct). Repeated once.

3.10 Testing of viral proteins in primary cells

The next step was to take these two viral proteins forward and to test them in primary cells (PBMCs). One additional observation from these results was that

the anti-HLA-A/B/C-APC mean fluorescence intensity (MFI) was not very high for the non-transduced (NT) cells, so the antibody was titrated and the transduction was repeated in three donor PBMCs. **Figure 19** shows a representation of FACS staining for one donor. The constructs used in this experiment were ones that contained US11, ICP47 and siRNA_Outside_01. The siRNA construct was used as a control.

These results demonstrate that HLA Class I can be knocked down from primary cells with a high level of efficacy, and that the previous results obtained from the modified K562 cell line can be disregarded. PBMCs transduced with the ICP47 and US11 constructs show a high level of HLA Class I knockdown; both seeming to be similar in the level of knockdown. The siRNA construct was ineffective in the donors. Testing of constructs was repeated in PBMCs from eight donors, in order to demonstrate reproducibility regardless of the donor HLA haplotype. Data was collated by calculating percentage of HLA Class I expression relative to the non-transduced cells, before being averaged and presented in **Figure 20**. The reason for the lack of knockdown by the strategies targeting β2m is possibly due to the high levels of endogenous β2m present in the cell, meaning that the effect of the constructs was negligible in comparison to the amount of β2m. The β2m strategies are also competitive strategies, competing with the endogenous β2m present in the cell. The two viral proteins act by non-competitive mechanisms, with ICP47 acting as a physical block to peptide entry into the ER and US11 binding to immature MHC Class I molecules and ubiquitinating them, regardless of the presence of β2m.

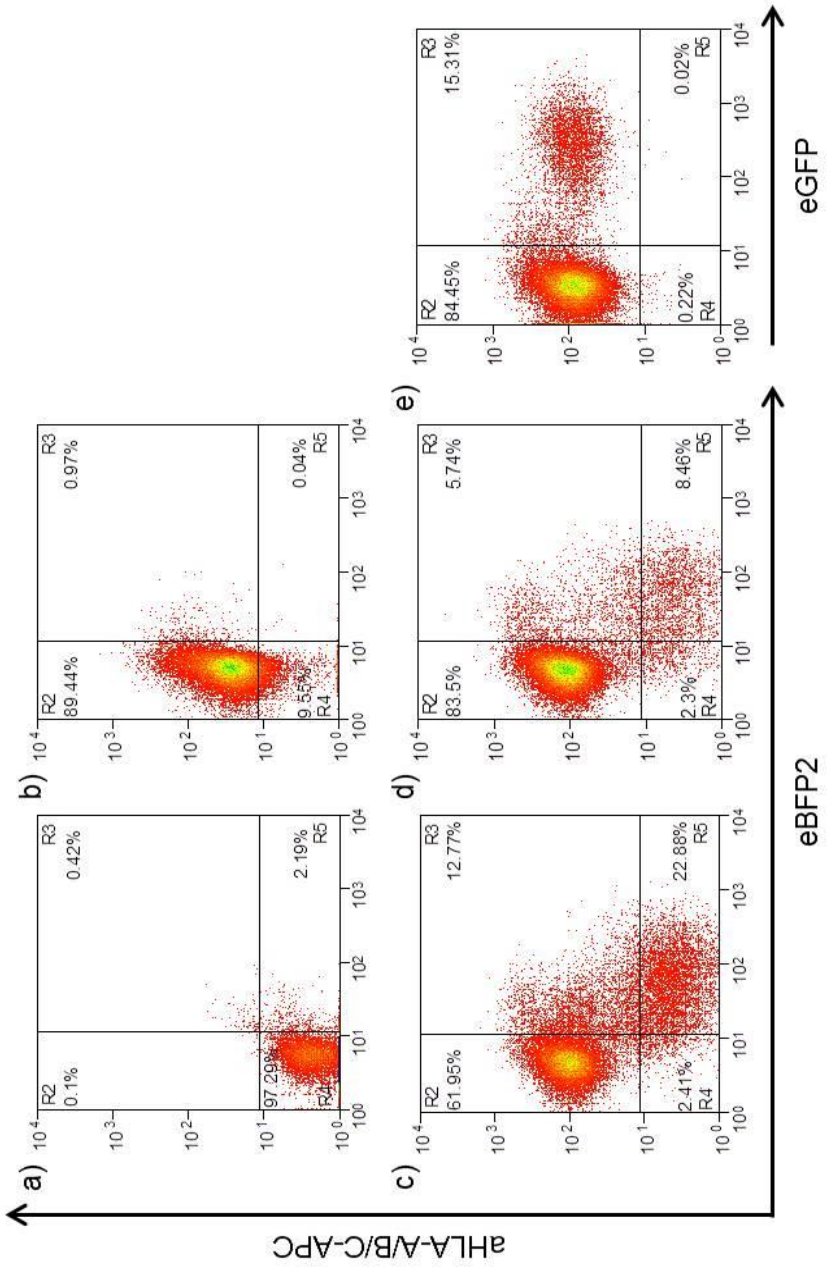


Figure 19 – Testing of HLA knockdown constructs in PBMCs. a) NT without antibody b) NT with antibody c) ICP47 d) US11 e) siRNA Outside 01. Antibody used was anti-HLA-A/B/C-APC (BD). Marker gene for ICP47 and US11 is eBFP2; siRNA Outside 01 eGFP. Sample representative of all donors tested (see **Figure 20**).

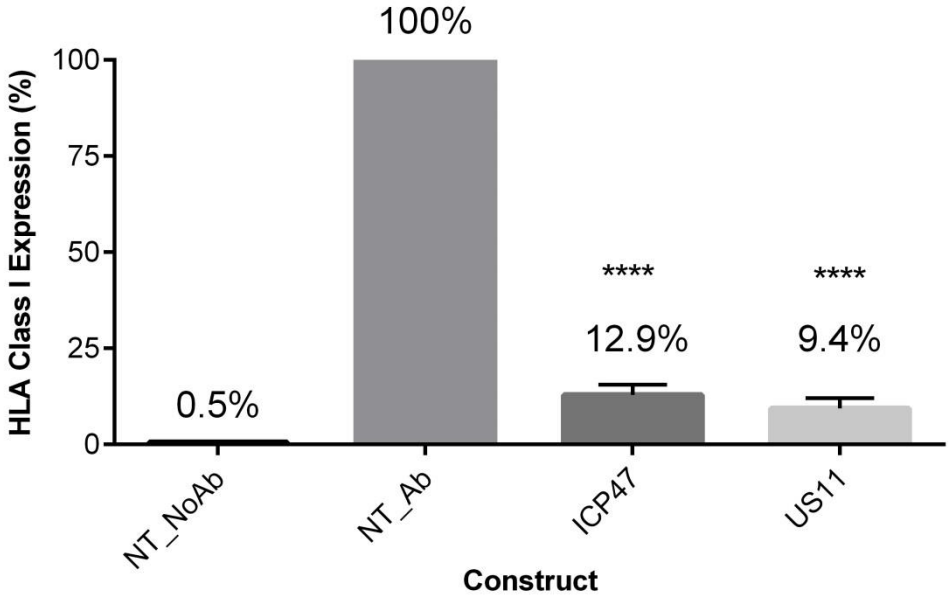


Figure 20 – Transduction of PBMCs with HLA Class I knockdown constructs, ICP47 and US11. Graph shows percentage of HLA Class I expression when compared to NT cells with Ab – set as 100%. (NT – non-transduced; NoAb – No antibody used; Ab – antibody used). Ten donors shown, one-way ANOVA used to calculate statistical significance ($p<0.0001$). Error bars show S.E.M.

These results show that it is possible to achieve knockdown of 87% with ICP47 and 90% with US11. It was unknown as to whether this level of knockdown was sufficient to prevent alloreactivity in an *in vivo* context. In order to achieve the highest level of HLA Class I knockdown possible, the next step was to combine these two proteins to determine if their co-expression could increase HLA Class I knockdown further. In order to do this, the viral proteins were expressed in one construct, separated by the 2A proteinase (**Figure 21**).

Figure 21 – Design of double-viral protein transgenes. ICP47-2A-US11 (top) and US11-2A-ICP47 (bottom) transgenes shown. Blue arrows indicate oligonucleotide primers used to amplify fragments from template DNA. Multi-coloured blocks denote amino acid sequence.

The 2A proteinase is derived from the foot-and-mouth disease virus (FMDV) and is able to ‘self-cleave’ at its own C terminus. In proteolytic processing, the primary 2A/2B cleavage of the aphthovirus and cardiovirus is used to

cleave polyproteins (Donnelly et al. 1997). The 2A peptide is very short, around 18 amino acids long, and along with the proline residue located at the N terminus of the 2B protein, forms an element capable of autonomous cleavage (Donnelly et al. 2001). There are multiple naturally occurring versions of the 2A peptide, mostly originating from viruses. The one used in this project was the '2A-like' peptide derived from the insect virus TaV (Pringle et al. 1999). The advantage of using the TaV 2A-like peptide over the FMDV 2A peptide is that it is shorter, and also has a higher level of cleavage activity (>99% compared to ~90% for FMDV 2A) (Donnelly et al. 2001). This peptide was cloned into an SFG vector, flanked by the two viral proteins. In one of the vectors, the orientation of viral proteins was reversed, resulting in the following vectors: SFG.ICP47-2A-US11 and SFG.US11-2A-ICP47. Both vectors had eBFP2 as a marker gene with an internal ribosomal entry site (IRES) sequence. Correct assembly was confirmed by sequencing and retrovirus was generated for both constructs before transduction into PBMCs from two donors (**Figure 22**).

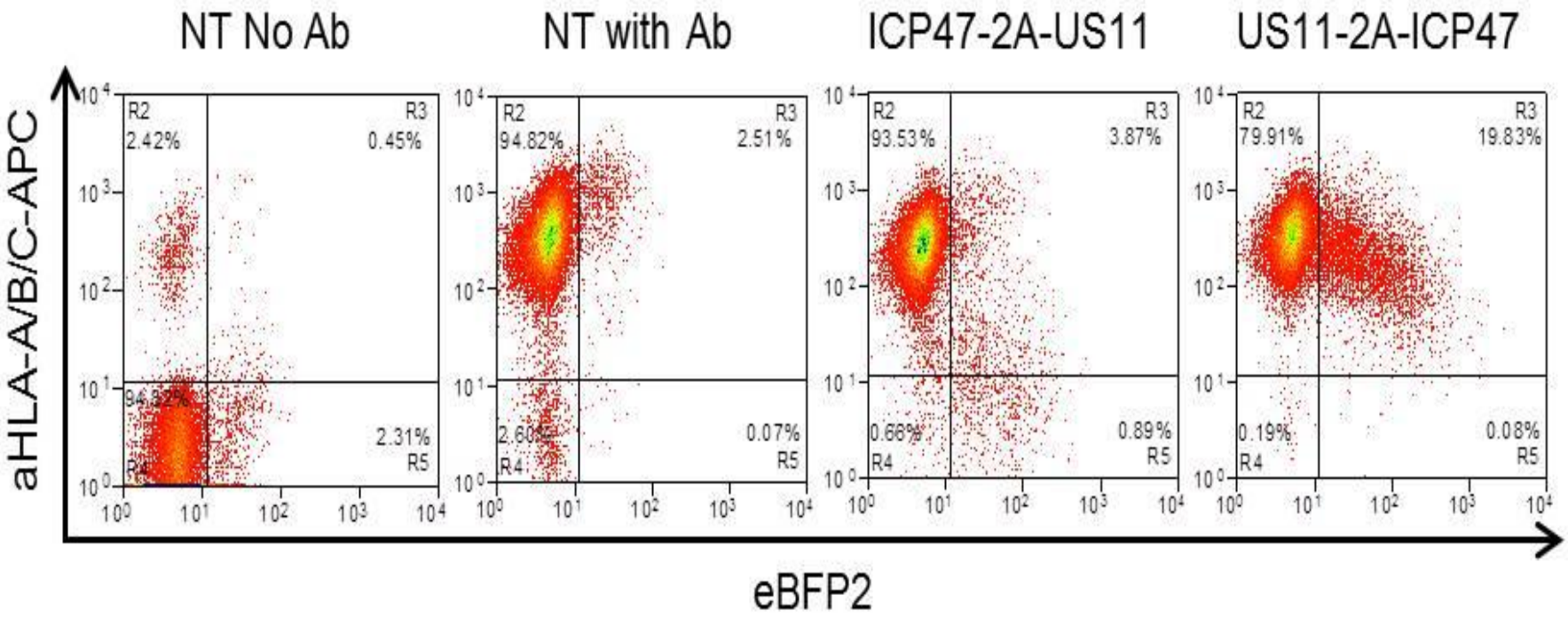


Figure 22 – Double viral protein constructs tested in donor PBMCs (one donor shown as representative of both donors tested). Marker gene used was eBFP2. (NT – non-transduced; Ab – Antibody). Performed once.

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Although the transduction efficiency was low in the cells transduced with the ICP47-2A-US11 construct, it can clearly be seen that this construct is more effective at achieving HLA Class I knockdown compared to US11-2A-ICP47. This suggests that the N terminus of the 2A peptide is inhibiting the activity of the attached protein, but the C terminal proline residue does not have any effect on activity. The 2A peptide self-cleaves at the C terminus, thereby allowing separate expression of both proteins. As a result of the cleavage event, an extra proline residue is added to the N terminal of the viral protein at the C terminal end of the 2A peptide and nineteen residues are added to the C terminus of the first viral protein. This may have a detrimental effect on the expression of the US11 viral protein in the US11-2A-ICP47 construct. If further investigation was required, the inclusion of a Furin cleavage site at the N terminal end of the 2A peptide could be used. As the level of HLA Class I knockdown did not appear to be anymore significant than that previously seen with the individual constructs, only one viral protein was taken forward in this project. Due to US11 being slightly more effective at HLA Class I knockdown than ICP47, this protein was the one taken forward for further testing in cell lines and functional assays.

3.11 Addition of sort-suicide gene into HLA Class I knockdown construct

As the transduction of US11 and ICP47 as separate constructs was successful, the next step was to introduce a way of selecting the transduced cells. Traditionally, cell sorting has been done based on the expression of a fluorescent marker such as BFP or GFP. A member of Dr Pule's group, Brian Philip, has created a novel sort-suicide gene that combines the minimal epitopes of the CD20 and CD34 antigens, resulting in a construct that is around 136 amino acids in length. This protein is expressed on the cell surface and comprises of two CD20 epitopes and one CD34 epitope, protruded from the cell surface by a CD8 stalk. The CD20 epitopes are recognised by the CD20 mAb, Rituximab, thereby providing a 'suicide' mechanism by which to

deplete the modified cells. The CD34 epitope is recognised by a CD34 antibody (QBend10 clone). The expression of the CD34 epitope acts as a marker gene, allowing for magnetic selection of transduced cells using Miltenyi MACS aCD34 beads (Philip et al. 2014).

3.11.1 Design for creating RQR8-2A-HLA_knockdown constructs

The constructs were created using PCR as described previously, resulting in two constructs, SFG.RQR8-2A-ICP47 and SFG.RQR8-2A-US11 (**Figure 23**). Correct assembly was verified by restriction enzyme digest and sequencing.

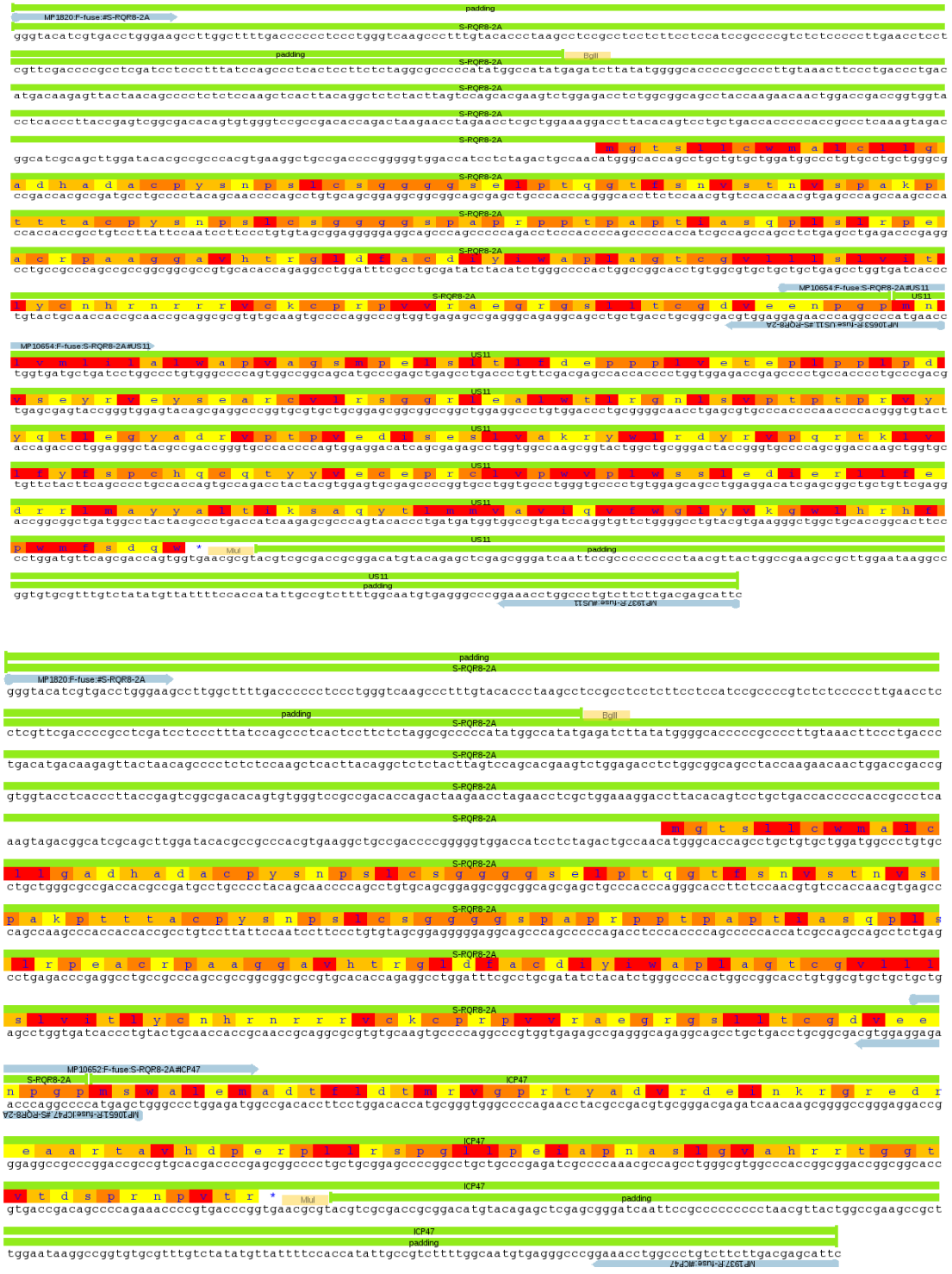

Figure 23 – Construct design for RQR8 assembly with the HLA knockdown viral proteins. Top half shows design for SFG.RQR8-2A-US11; bottom half shows design for SFG.RQR8-2A-ICP47. Blue arrows indicate oligonucleotide primers used for amplification of fragments from template DNA; multi-coloured blocks denote amino acid sequence.

3.11.2 Transduction and MACS sorting of RQR8-2A-HLA_knockdown constructs into SupT1s

After assembly of the above constructs, retroviral supernatant was generated and used to transduce SupT1s (**Figure 24**). The RQR8 sort-suicide gene was used as the marker gene in FACS analysis. SupT1s that had been transduced were also sorted on their expression of the RQR8 sort-suicide gene. aCD34 MACS beads (Miltenyi) were used to magnetically sort transduced cells. As can be seen, the magnetic selection process is very efficient, giving purity of 95% for the US11 construct and 99% for the ICP47 construct (**Figure 25**). It is also clear that while both proteins are able to knockdown MHC Class I expression on SupT1s and PBMCs by around 90%, US11 seems to be slightly more effective at this than ICP47. Therefore, the preferred construct to use would seem to be the one containing the US11 viral protein.

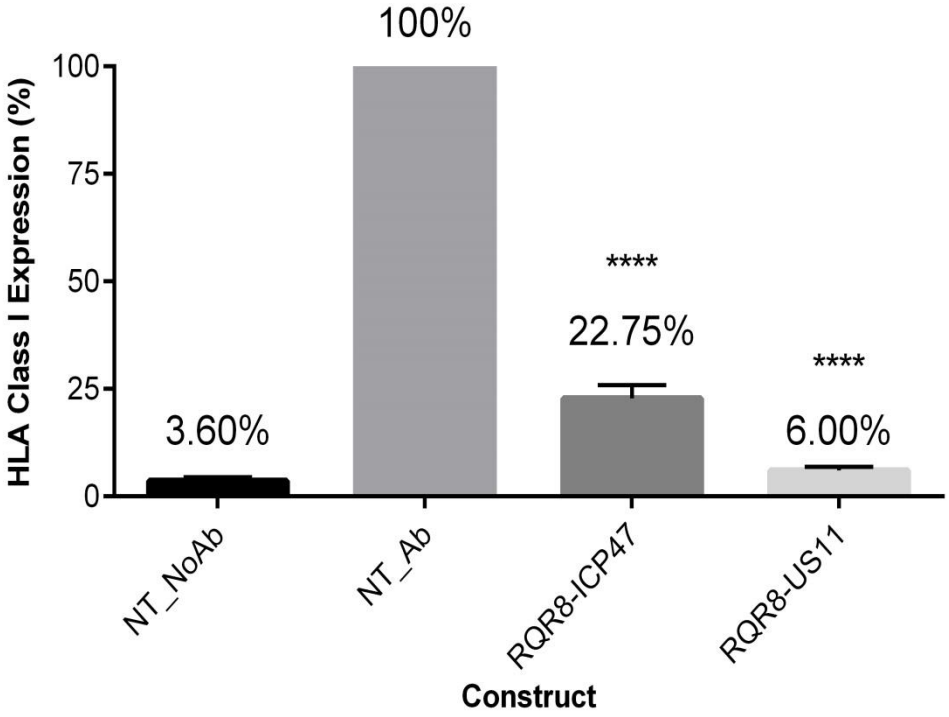

Figure 24 - Transduction of SupT1s with constructs containing HLA knockdown proteins and RQR8 sort-suicide gene. Graph shows percentage of HLA Class I expression when compared to NT cells with Ab – set as 100%. (NT – non-transduced; NoAb – No antibody used; Ab – antibody used). Eight repeats shown, one-way ANOVA used to calculate statistical significance ($p<0.0001$). Error bars show S.E.M.

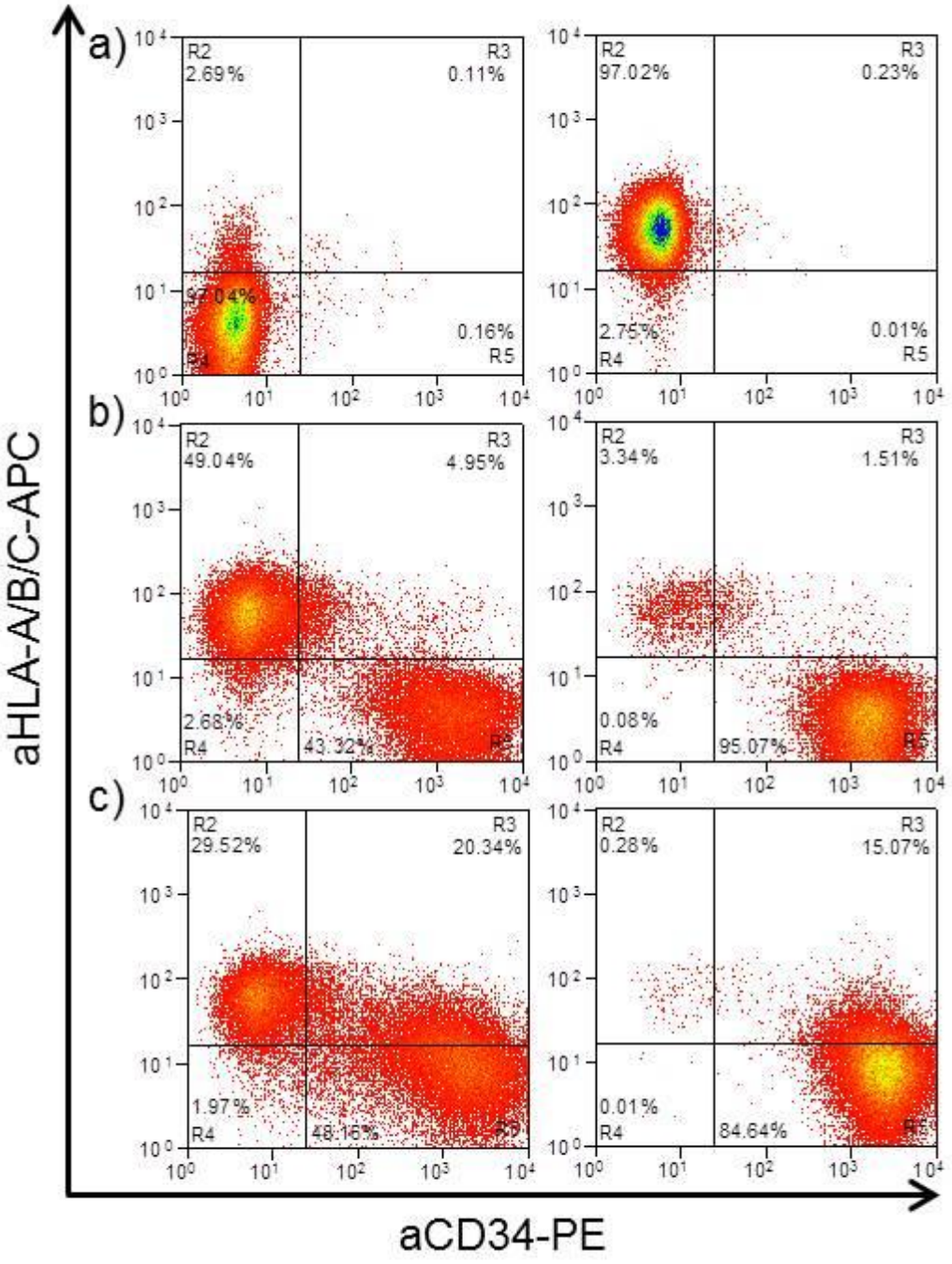

Figure 25 - HLA knockdown constructs with RQR8 gene transduced into SupT1 cells followed by a CD34-MACS bead sort. a) NT SupT1s (LHS – No Ab; RHS – with Ab). b) SupT1s with RQR8-US11 construct. c) SupT1s with RQR8-ICP47 construct. For b) and c), LHS shows unsorted cells, RHS shows cells after MACS sort. NT – non-transduced; Ab – Antibody. Repeated once.

3.11.3 Transduction and MACS sorting of RQR8-2A-HLA_knockdown constructs into PBMCs

Due to the success achieved in the SupT1 cell line, the experiment was repeated in primary cells (PBMCs) derived from healthy donors, using the RQR8 sort-suicide gene as the marker gene (**Figure 26**). **Figure 27** shows collated data from four donors.

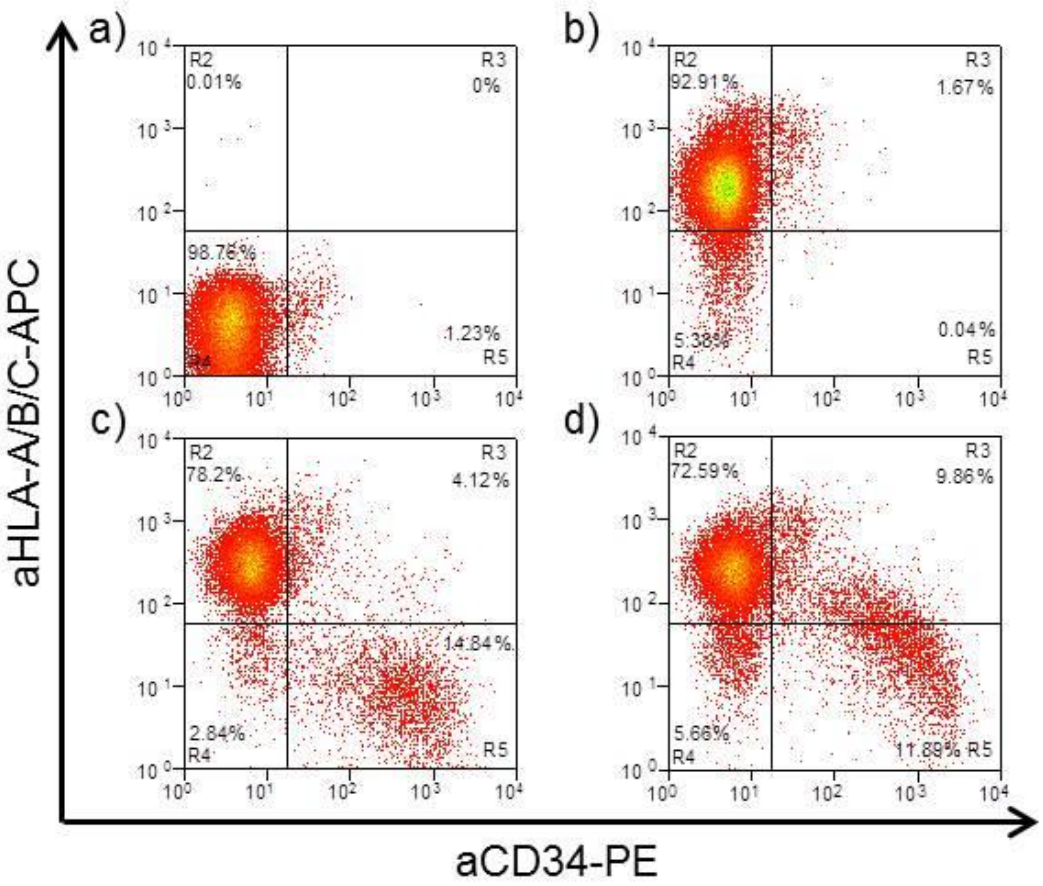

Figure 26 – Transduction of PBMCs with constructs containing HLA knockdown proteins and RQR8 sort-suicide gene a) NT PBMCs with no antibody added b) NT PBMCs with aHLA-A/B/C-APC and aCD34-PE antibodies added c) PBMCs transduced with SFG.RQR8-2A-US11 d) PBMCs transduced with SFG.RQR8-2A-ICP47 (NT – non-transduced).

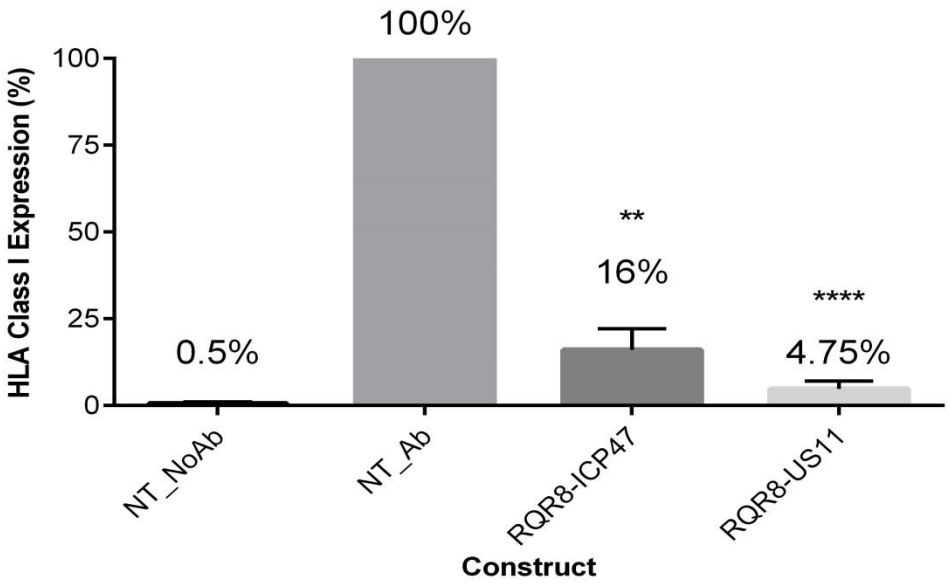

Figure 27 – Transduction of RQR8 HLA knockdown constructs in PBMCs. Graph shows percentage of HLA Class I expression when compared to NT cells with Ab – set as 100%. (NT – non-transduced; NoAb – No antibody used; Ab – antibody used). Four donors shown, one-way ANOVA used to calculate statistical significance ($p<0.0001$). Error bars show S.E.M.

It appears from this experiment that the 2A peptide is not adversely affecting expression of the two molecules. In a further repeat of this experiment, after transduction, the cells were sorted using aCD34 magnetic beads (Miltenyi) (**Figure 28**).

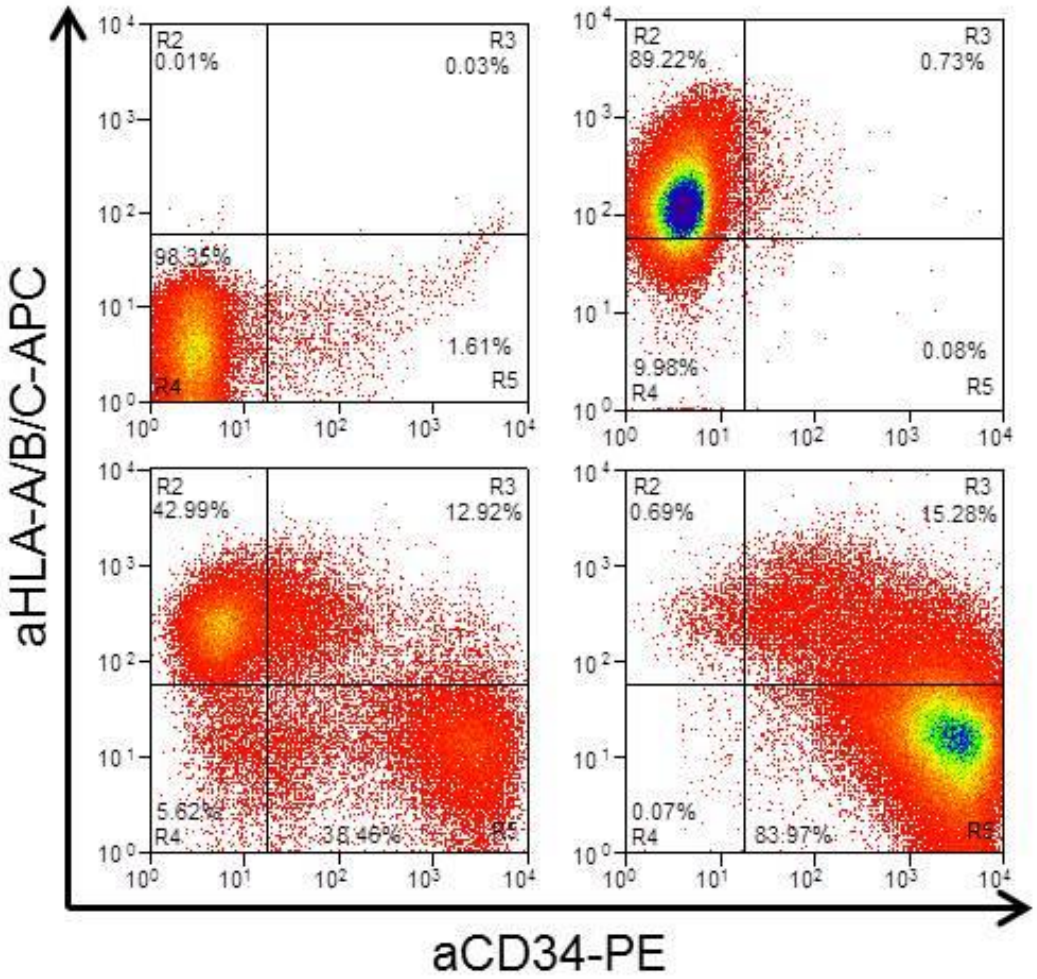

Figure 28 – Clinical grade sorting of donor PBMCs transduced with SFG.RQR8-2A-US11 construct. Top row – NT PBMCs without (LHS) and with (RHS) antibody added; second row – SFG.RQR8-2A-US11 transduced PBMCs. LS shows pre-sorted cells, RHS shows cells after aCD34 MACS bead sort. (NT – non-transduced). Repeated three times but statistical analysis not possible due to FACS analysis being performed on multiple machines with incompatible software.

As can be seen, the magnetic selection enables the effective purification of the transduced cells to a high level of purity. This experiment was repeated in multiple donors, with FACS analyses performed on different flow cytometers (CyAn, LSRFortessa, FACSVerse), with similar results obtained from all donors.

3.12 Testing the function effect of viral HLA knockdown proteins using a mixed lymphocyte reaction

It is clear that the two viral proteins are effective at achieving HLA Class I knockdown, and that they can be combined with the RQR8 sort-suicide gene, allowing for clinical grade selection of the transduced cells. Despite the high levels of knockdown (>90%), it is unknown whether this is sufficient to prevent an allogeneic response in an *in vivo* context. In order to attain an idea of this, an MLR was carried out, using PBMCs transduced with the SFG.RQR8-2A-US11 construct as targets and allogeneic PBMCs as the effectors. As US11 gave the higher levels of HLA Class I knockdown, this was the construct used in the MLR.

The transgenic cells, containing the HLA Class I knockdown construct were positively sorted using aCD34 MACS beads (Miltenyi) (as seen in **Figure 28**), before being irradiated (30Gy) and plated at various effector:target ratios (0.5:1 to 8:1). Effector PBMCs were CD56 depleted in order to remove NK cells from the co-culture, which would otherwise increase the level of killing due to NK-mediated null cell killing. The CD56 depletion was carried out by a negative selection of CD56 positive cells using aCD56 MACS beads (Miltenyi). The cells were co-cultured for five days, with tritiated thymidine (^{3}H) being added on the fourth day, allowing for 20hrs of uptake (**Figure 29**).

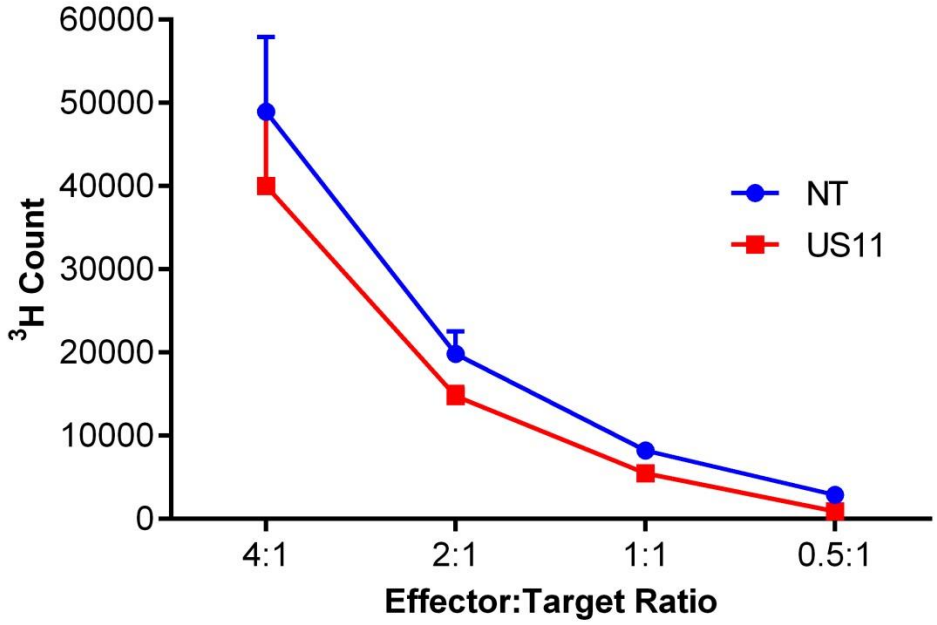

Figure 29 – MLR using two mis-matched donors. Either NT or US11-modified target cells were irradiated (30Gy) before being cultured with HLA mis-matched effector PBMCs at the indicated ratios. Chart legend shows targets – NT – non-transduced donor PBMCs. US11 – donor PBMCs transduced with RQR8-2A-US11 construct and positively sorted with aCD34 MACS beads. Effector PBMCs were negatively selected using aCD56 MACS beads. Cells were cultured for five days with ^{3}H added on the fourth day for 20hrs. Performed once in triplicate. Error bars show S.E.M.

From this result, it can be seen that the US11 viral protein has reduced the proliferation of the mis-matched effector PBMCs compared to the non-transduced PBMCs. What is also clear is that it does not completely prevent the proliferation of the effector PBMCs. What needed to be shown was how much of the proliferation of the effector PBMCs was due to incomplete HLA Class I knockdown and how much was due to HLA Class II. In order to do this, another MLR was carried out with the same donors used for the target and effector PBMCs as the MLR shown in the above figure. Additional controls were used, including the use of pan-anti-HLA Class I (derived from the W6/32 hybridoma) and pan-anti-HLA Class II (derived from the CR3/43 hybridoma) blocking antibodies (Barnstable et al. 1978; Naiem et al. 1981). The target

cells were incubated with the HLA blocking antibody/antibodies for 30minutes at room temperature in the dark before being irradiated (**Figure 30**).

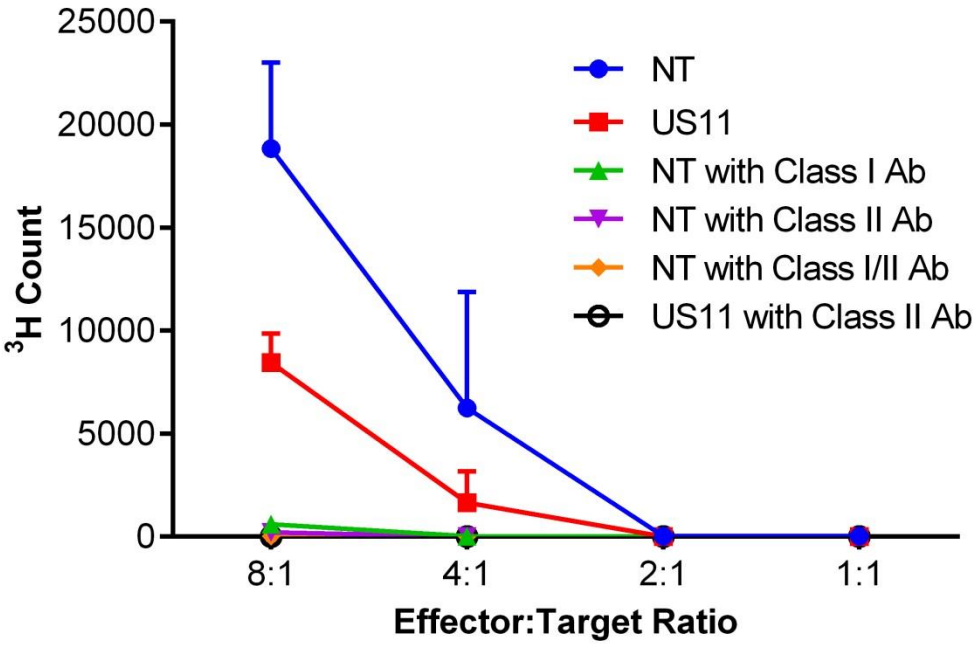

Figure 30 – MLR using mis-matched donor PBMCs (same donors as in Figure 26). Legend shows target populations used. NT – non-transduced; US11 – SFG.RQR8-2A-US11 construct used; Ab – antibody; Class I Ab – pan-anti-HLA-Class I antibody derived from W6/32 hybridoma; Class II Ab – pan-anti-HLA-Class II antibody derived from CR3/43 hybridoma. Error bars show S.E.M. Performed once in triplicate.

This result shows that the viral protein US11 reduces the effector cell proliferation by around half. The pan-anti-HLA Class I antibody used in this experiment show that there may be some reactivity due to the HLA Class I knockdown not being complete.

3.13 Conclusions

In conclusion, novel constructs have been generated that have been shown to knockdown the expression of HLA Class I from the surface of both cell lines and primary cells. Initially, fifteen different constructs were tested in the attempt to achieve knockdown of HLA Class I. These included targeting β2m

at the mRNA level and protein level, as well as targeting the HLA Class I molecule at the protein level by trying to disrupt the trafficking of the HLA molecule to the cell surface. The two most successful strategies used were derived from HSV (ICP47) and HMCV (US11). These two viruses have adapted to avoid the immune surveillance of the host organisms that they infect, with one aspect being to down-regulate the HLA expression in infected cells to prevent recognition of foreign peptides by the host immune system. The ICP47 and US11 proteins are ones that are designed to carry out this function, and this is the most likely reason why they have been the most successful strategies, with knockdown of 88% and 91% respectively.

Unfortunately, the modification of the K562 cell line to express HLA-A*0201, so that the model of HLA Class I knockdown in a cell line could be further verified, was unsuccessful as the level of HLA-A*0201 expression was minimal and transduction with the viral protein constructs did not provide any firm conclusions from an engineered cell line.

Despite this, the two viral proteins were very successful at knockdown of HLA Class I in primary cells, replicating the knockdown shown in the SupT1 cell line. When combining both viral proteins into one construct, the cleavage of the FMDV 2A peptide left an additional nineteen residues on the N-terminal protein, which impeded the activity of US11 when the construct was orientated with US11 as the N terminal protein. The remaining proline residue left on the C terminal protein had no apparent effect on expression and activity. In the reverse orientation, with US11 as the C terminal protein and ICP47 as the N terminal protein, the activity of the two proteins together was no more increased than when using US11 on its own. These results suggest that while both proteins are capable of causing HLA Class I knockdown individually, combining the two proteins into one construct would be inefficient and would reduce the remaining vector capacity when introducing the other strategies.

It was possible, though, to combine the viral proteins with the sort-suicide gene using the 2A peptide. The proline residue left on the N terminal protein, in this case, the RQR8 sort-suicide gene, had no apparent effect on expression, and therefore it was possible to have dual expression of both proteins using just

one construct. When transduced into the SupT1 cell line, it was clear that there was no reduction in effect of the viral proteins with the addition of the sort-suicide gene. It was also demonstrated that it is possible to sort the transduced cells based on their expression of the RQR8 sort-suicide gene. The RQR8-2A-HLA knockdown constructs were then taken forward into primary cells and shown to have a very similar activity to their activity in SupT1 cells. The RQR8-2A-US11 construct was shown to cause a reduction in proliferation of effector cells in a mis-matched donor context, suggesting that it would be possible to use this construct as part of the overall strategy. Another experiment that may be valuable to perform would be to use $CD8^+$ T cells as effectors to determine if HLA Class I negative cells avoid activating this population.

3.14 Final conclusions

- Successful testing of various strategies aimed at causing HLA Class I knockdown.
- Two strategies clearly more successful than the other strategies tried – the HSV protein ICP47 and the HMCV protein US11.
- Both ICP47 and US11 result in high levels of HLA Class I knockdown.
- Combination of viral proteins with RQR8 sort-suicide gene allows for clinical grade sorting of modified cells by MACS sorting. This has been shown in a cell line and in primary cells.
- Primary cells that have been transduced with US11 cause a reduction of proliferation of allogeneic effector cells as shown through MLR.

Chapter Four: HLA-G expression

4.0 Aims

- To determine a method of allowing HLA Class I negative cells to avoid NK cell killing.
- To extract HLA-G from genomic DNA to mimic HLA-G expression by trophoblasts.
- To test the effect of HLA-G expression on NK cell function.

4.1 Introduction

In healthy human tissues, HLA-G gene transcription is present in most cells, but this is only translated in certain tissues, including the trophoblast, pancreas and thymus. In diseased tissues, HLA-G expression can be found in viral infections, malignancies and autoimmune diseases (Carosella et al. 2003). In diseased cells and tissues, HLA-G can be found to be expressed by both the unhealthy cells and also the infiltrating immune cells, suggesting that HLA-G has a role in immune responses and in stressed microenvironments (Carosella et al. 2008).

As previously discussed, HLA-null cells are vulnerable to cell killing by NK cells. NK cells require recognition of self MHC class I molecules in order for the inhibition of the release of lytic granules from the immunological synapse (Bryceson et al. 2011). HLA molecules interact with inhibitory receptors on the NK cell, so without this interaction, NK cell activation is not inhibited. Despite this, there are some forms of cancer that are HLA class I negative, yet they avoid killing by NK cells (Menier et al. 2008). Studies have shown that cancers such as AML, ALL and B-cell CLL express HLA-G and avoid killing by NK cells (Nückel et al. 2005; Mizuno et al. 2000; Poláková et al. 2003). In pregnancy, various isoforms of HLA-G are used by trophoblasts, to prevent NK cell killing (Hunt & Langat 2009). There are seven isoforms of HLA-G. Isoforms one to four are membrane-bound; isoforms five to seven are soluble versions of isoforms one, two and four respectively. In comparison, as of the beginning of

2013, HLA-A has 2995 variants and HLA-C has 2553 variants on the IMGT/HLA database (Robinson et al. 2013). HLA-G1 and HLA-G5 are the most studied isoforms, with both having the same extracellular structure: a three domain heavy chain able to associate with β2m and a nonamer. The other isoforms have fewer globular heavy chains and therefore should not be able to associate with β2m. (Carosella et al. 2008).

Whilst not reported to have significant immune stimulatory properties, HLA-G has been shown to have other functions other than the inhibition of NK cells. The immunoglobulin-like transcript (ILT)-2, ILT4 and KIR2DL4 receptors all bind to HLA-G. ILT2 is expressed on some B and T cells, all monocytes, and NK cells. ILT4 is expressed only on monocytes and dendritic cells (DCs), and KIR2DL4 is expressed on $CD56^{bright}$ NK cells. ILT2 and ILT4 are inhibitory receptors, whereas KIR2DL4 has both an ITIM in its cytoplasmic domain and a positively charged arginine residue in the TMD (Selvakumar et al. 1996; Yusa et al. 2002). In this project, it was the ability of HLA-G to inhibit NK cells that was investigated.

4.2 Strategy to extract HLA-G from genomic DNA

The aim was to replicate the inhibition of NK cells by HLA-G to avoid NK cell killing of the null T cells by introducing a membrane-bound isoform of HLA-G, HLA-G1, into the retroviral cassette. The aim was to pull out HLA-G from genomic DNA using primers designed by Dr Pule. The reference sequence for HLA-G was taken from the NCBI database - ENST00000360323 (NCBI 2011a). Oligonucleotides were designed to bind to the exon junctions of the HLA-G sequence to enable it to be cloned from genomic DNA. One strategy was to have HLA-G on its own in one vector; the second was to have HLA-G joined by a peptide linker to β2m (**Figure 31** – **Figure 32**). **Figure 33** shows a pictorial representation of how the β2m-Linker-HLA-G construct might assemble and fold correctly upon expression. Genomic DNA was obtained from donor PBMCs and then used as the template for HLA-G. We already had a vector containing β2m which was used as a template.

4.2.1 Design of HLA-G constructs

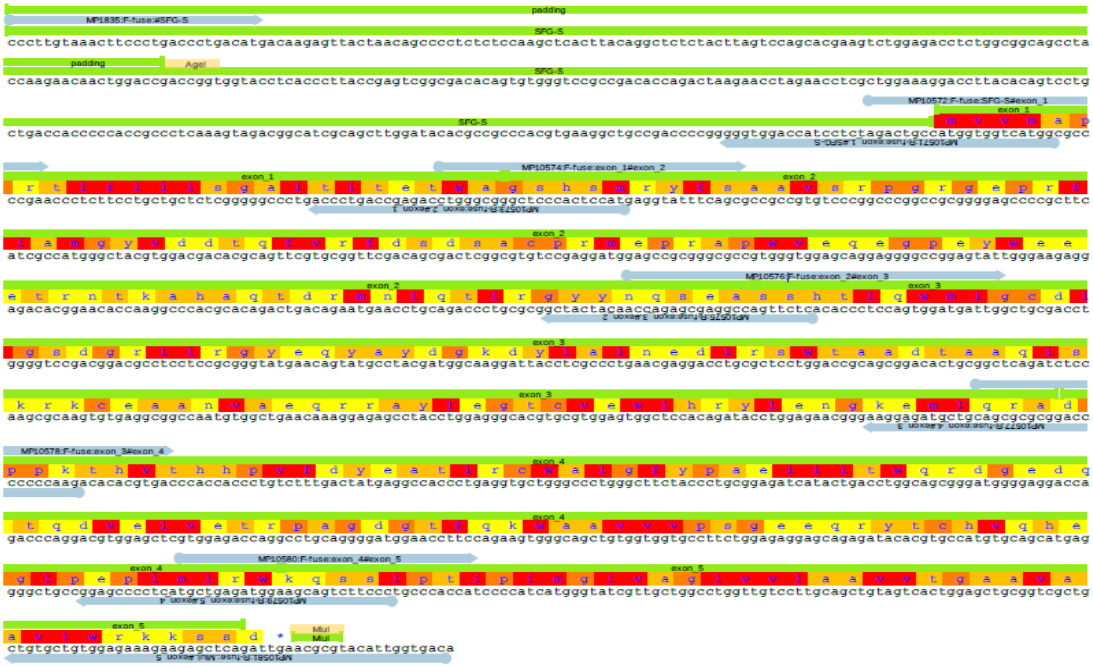

Figure 31 – Construct design for assembly of HLA-G into an SFG vector. Template DNA for HLA-G was derived from genomic DNA.

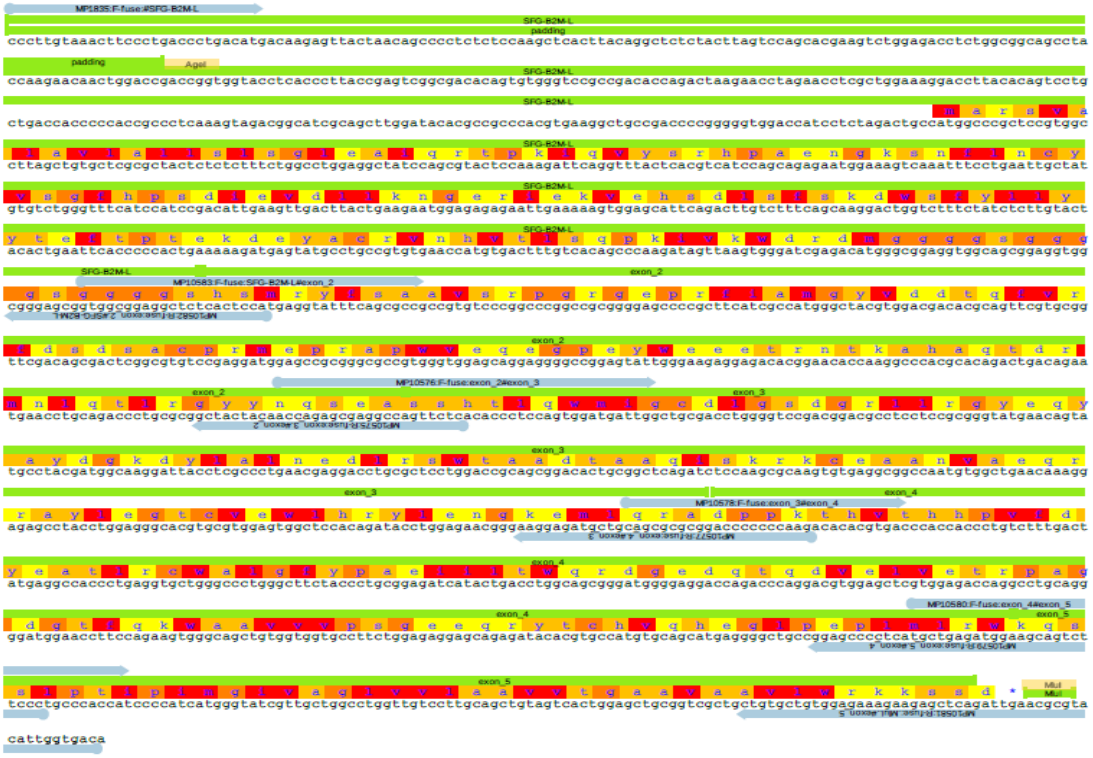

Figure 32 - Construct design for assembly of β2m-L-HLA-G into an SFG vector. Template DNA for HLA-G was derived from genomic DNA.

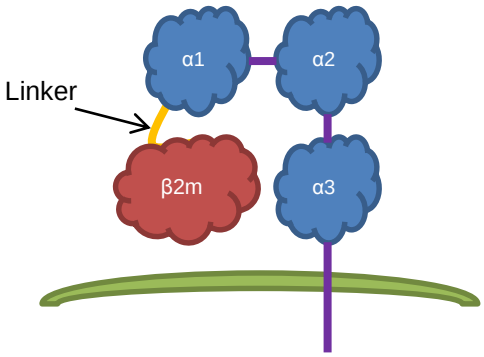

Figure 33 – Schematic of assembly of β2m-Linker-HLA-G construct. Linker (in yellow) is postulated to allow orientation and correct assembly of full construct, without reliance on the presence of endogenous β2m.

4.2.2 HLA-G construct repair

Unfortunately, after cloning of these two constructs, errors were found in the sequencing. In the HLA-G alone construct, there were multiple errors resulting in point mutations. In the β2m-L-HLA-G construct, there was one silent mutation which was discovered to be a SNP (NCBI 2011b) and a single nucleotide mutation, resulting in an amino acid substitution. In order to repair these constructs, it was decided to repair the amino acid mutation and to then use this as the template to clone out the HLA-G alone construct. Again, this strategy was carried out using oligonucleotide primers; correct repair and construction was verified by sequencing. **Figure 34** shows the sequence obtained after extraction from genomic DNA and the subsequent repair. The only difference between the ENST00000360323 sequence and the cloned sequence was a G>A alteration in a Proline codon, resulting in no amino acid change.

ATGAGGTATTTCAGCGCCGCCGTGTCCCGGCCCGGCCGCGGGGAGCCCCGCTTCATCGCC
ATGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGACTCGGCGTGTCCGAGG
ATGGAGCCGCGGGCGCCGTGGGTGGAGCAGGAGGGGCC**A**GAGTATTGGGAAGAGGAGACA
CGGAACACCAAGGCCCACGCACAGACTGACAGAATGAACCTGCAGACCCTGCGCGGCTAC
TACAACCAGAGCGAGGCCAGTTCTCACACCCTCCAGTGGATGATTGGCTGCGACCTGGGG
TCCGACGGACGCCTCCTCCGCGGGTATGAACAGTATGCCTACGATGGCAAGGATTACCTC
GCCCTGAACGAGGACCTGCGCTCCTGGACCGCAGCGGACACTGCGGCTCAGATCTCCAAG
CGCAAGTGTGAGGCGGCCAATGTGGCTGAACAAAGGAGAGCCTACCTGGAGGGCACGTGC
GTGGAGTGGCTCCACAGATACCTGGAGAACGGGAAGGAGATGCTGCAGCGCGCGGACCCC
CCCAAGACACACGTGACCCACCACCCTGTCTTTGACTATGAGGCCACCCTGAGGTGCTGG
GCCCTGGGCTTCTACCCTGCGGAGATCATACTGACCTGGCAGCGGGATGGGGAGGACCAG
ACCCAGGACGTGGAGCTCGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGG
GCAGCTGTGGTGGTGCCTTCTGGAGAGGAGCAGAGATACACGTGCCATGTGCAGCATGAG
GGGCTGCCGGAGCCCCTCATGCTGAGATGGAAGCAGTCTTCCCTGCCCACCATCCCCATC
ATGGGTATCGTTGCTGGCCTGGTTGTCCTTGCAGCTGTAGTCACTGGAGCTGCGGTCGCT
GCTGTGCTGTGGAGAAAGAAGAGCTCAGATTGA

Figure 34 – Final sequence coding for HLA-G extracted from genomic DNA and repaired as described. One base pair was different compared to the ENST00000360323 reference sequence (G>A), which is shown in red font.

4.3 HLA-G expression in cell lines

The HLA-G and β2m-HLA-G molecules were both cloned into SFG vectors containing the eBFP2 marker gene. Both the Jurkat cell line and PBMCs do not express HLA-G so the constructs were tested in these cells to determine levels of HLA-G expression achievable using these constructs (**Figure 35**). FACS analysis was performed, and demonstrated that HLA-G expression was possible in both cell lines. Interestingly, for both cell lines, there was a higher level of HLA-G expression after transduction of the HLA-G construct compared to the β2m-HLA-G construct. This may be as a result of the β2m molecule. The cell lines would normally produce β2m which associates with other HLA molecules. It is known that β2m associates with the HLA molecule inside the ER during folding and assembly, therefore having the β2m molecule linked to the HLA-G construct may result in inefficient folding and assembly in the ER (Marsh et al. 1999). **Figure 35** shows expression results from Jurkat cells and PBMCs from one donor. One donor has been used as a representative of all donors as FACS data was acquired on three different machines with different

fluorophores attached to the aHLA-G antibody, and therefore cannot be collated statistically.

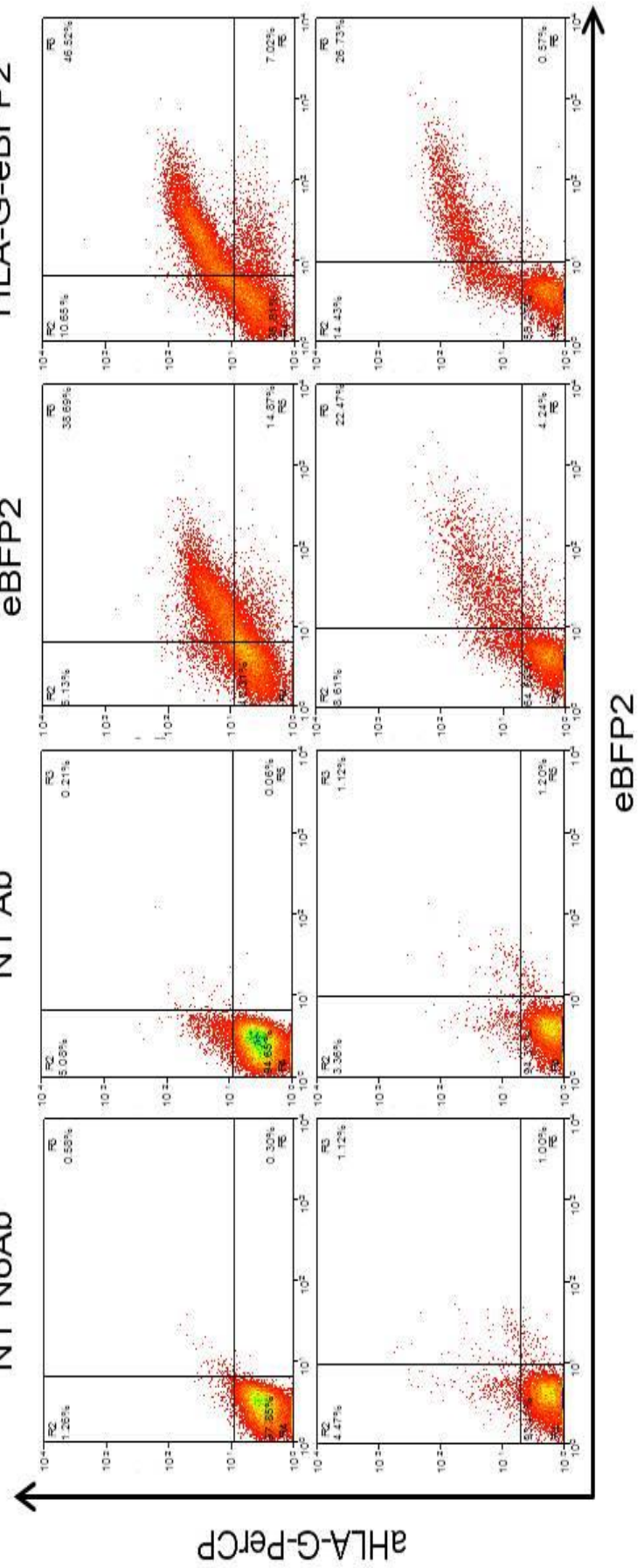

Figure 35 – HLA-G expression in transduced cell lines. Cells were transduced with constructs and stained with anti-HLA-G-PerCP antibody. Marker gene was eBFP2. Top row shows Jurkat cell line, bottom row shows PBMCs. NT – non-transduced. Repeated twice in several donors.

Statistics were gathered for the Jurkat, K562 and SupT1 cell lines. As staining levels varied between cell lines, a gate was set to exclude any cells expressing HLA-G below an MFI of 10. A second gate was used for the remaining cells, and the percentage of cells that were HLA-G positive was recorded (**Figure 36**). There were three replicates for the Jurkat and K562 cell lines and four replicates for the SupT1 cell line. Statistical significance can be seen in all cell lines transduced with the two HLA-G constructs when compared to the non-transduced cells, except for PBMCs (**Figure 37**).

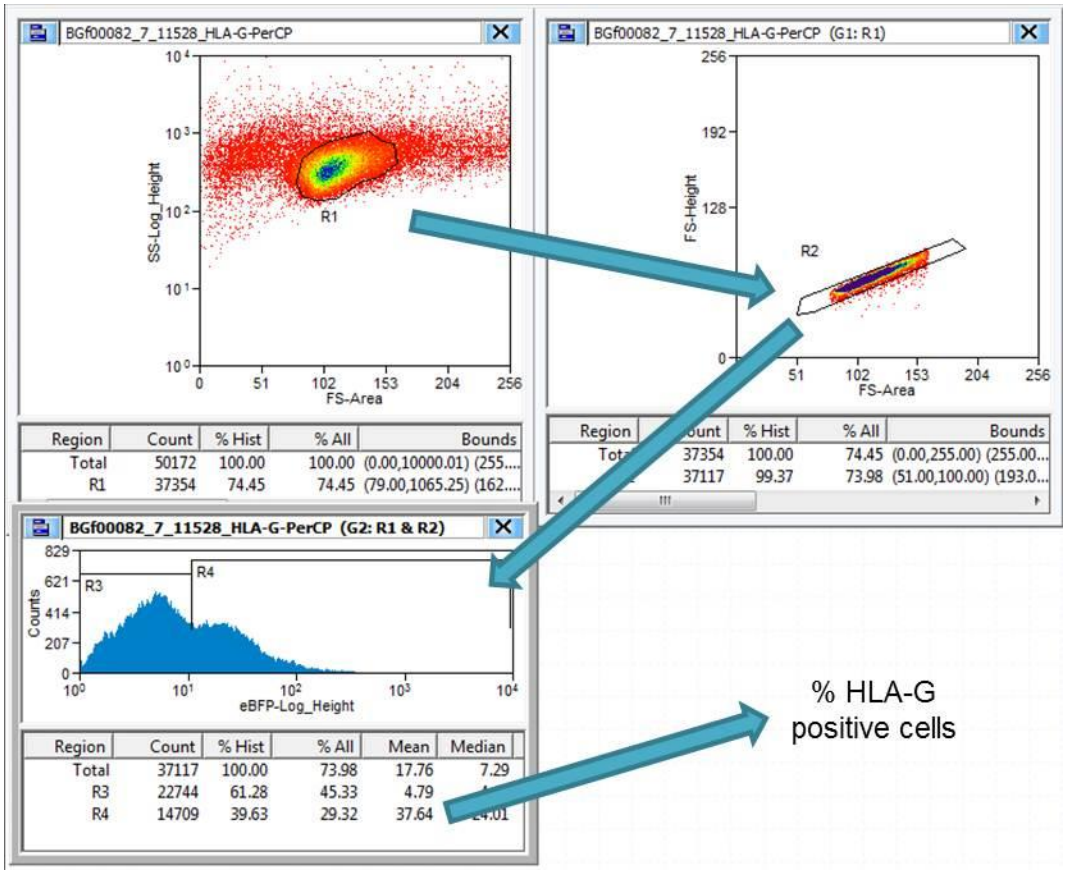

Figure 36 – Gating strategy to determine percentage of HLA-G positive cells. Gate R1 was gated onto the top right dot plot to remove debris and dead cells. R2 was used to exclude doublets and was gated onto the histogram in the bottom left panel. From this, cells with an MFI <10 were excluded (R3) and R4 was used to provide a value of HLA-G positive cells. Cells shown are Jurkat cells transduced with the HLA-G only construct. Representative of the gating strategy used for all cell types in **Figure 37**.

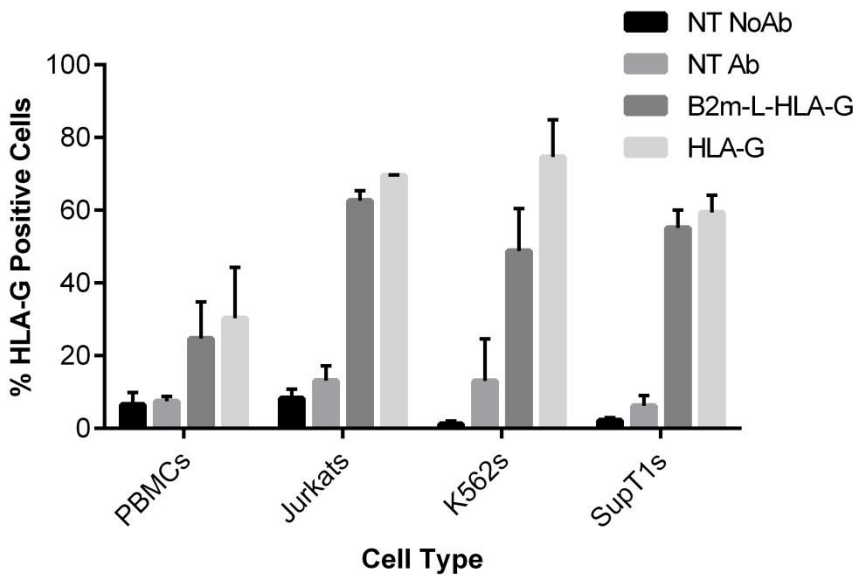

Figure 37 – Percentage of cells stained as HLA-G positive by aHLA-G-PerCP (Jurkat, SupT1 cell lines and PBMCs) or aHLA-G-PE (K562 cell line). Columns coloured according to construct used. Both constructs showed significant ($p<0.0001$) effects on HLA-G expression in Jurkat, K562 and SupT1 cell lines, but no significance shown in the PBMCs. NT – non-transduced; Ab – antibody. Performed twice in PBMCs, three times in Jurkat and K562 cell lines, four times in SupT1 cell line.

It can clearly be seen that the cell lines successfully express HLA-G, with the HLA-G alone construct giving slightly higher levels of expression (ns). HLA-G can also be expressed in PBMCs, but at a lower level in comparison to the cell lines. This will need to be optimised.

4.4 Co-expression of HLA-G with HLA knockdown strategies

One caveat that was thought of before proceeding with further functional work was to assess the stability of the HLA-G transgene expression when expressed with the HLAko constructs. As described previously, US11 causes HLA Class I knockdown by ubiquitination of the HLA Class I molecule in the ER, causing it to be retrotranslocated to the cytoplasm, where it is degraded in the proteasome. ICP47 functions by blocking peptide entry into the ER via the TAP protein. There is a possibility that both US11 and ICP47 may hinder

the expression of HLA-G. Although HLA-G is a non-classical HLA molecule, it does have a similar globular structure, with three alpha domains and it requires association with β2m to be expressed on the cell surface. As it has a similar structure, HLA-G may therefore be recognised by US11 and cause it to be removed from the ER in the same way as classical HLA Class I molecules. Additionally, if HLA-G has any antigen presentation functions, or if it requires an associated peptide in order to be presented on the cell surface, then ICP47 may also hinder peptide loading, resulting in low or poor HLA-G expression. Therefore, following expression of HLA-G in PBMCs, it was important to demonstrate that both the HLA knockdown strategies and the HLA-G molecules can be co-expressed.

This experiment was carried out in SupT1 cells as they express HLA Class I positive and are HLA-G negative. SupT1 cells were transduced with one of the two RQR8-2A-HLA knockdown constructs (ICP47 or US11) and one of the two HLA-G constructs (with eBFP2 marker gene) 72-96hours later (**Figure 38**). To allow for the possibility of the first construct hindering the expression of the second construct, both orders of transduction were tested (i.e. HLA knockdown construct transduction then HLA-G construct transduction or HLA-G transduction followed by HLA knockdown construct transduction). Cells were stained with the previously described antibodies, aHLA-A/B/C-APC and aHLA-G-PE. The aCD34 antibody was not included as the conjugates available at the time were unsuitable.

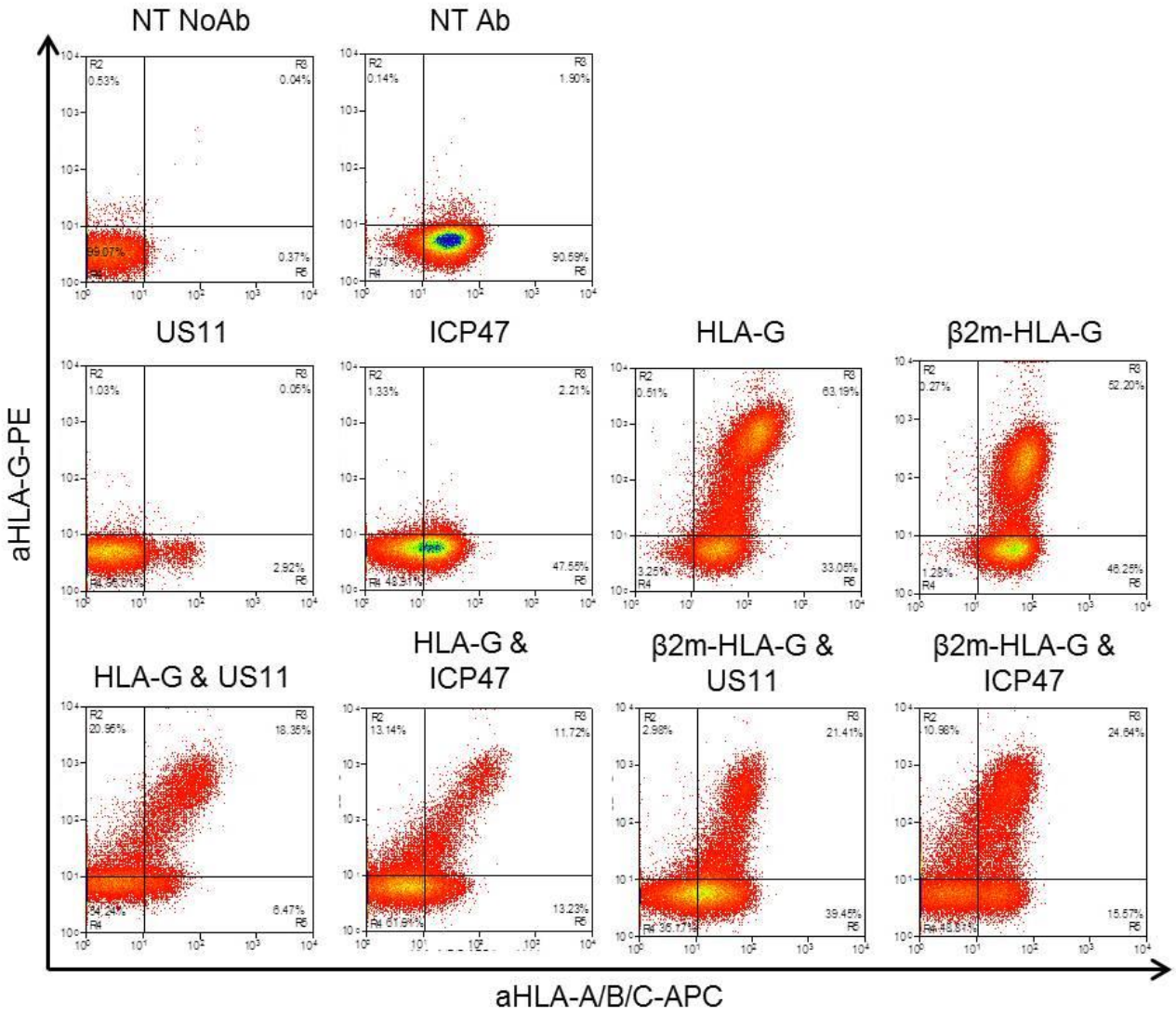

Figure 38 - HLA-G and viral protein co-expression in SupT1 cells. SupT1 cells were transduced with the viral HLA knockdown proteins ICP47 or US11; or the two HLA-G constructs (second row). Cells were then transduced again with the relevant constructs (third row). HLA-G was detected using anti-HLA-G-PE; HLA-A/B/C was detected with anti-HLA-A/B/C-APC. NT – non-transduced cells; Ab – antibody. Performed once.

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As can be seen, the HLA-G is well expressed in SupT1s. The issue with this experimental set up is that it appears that the aHLA-A/B/C antibody also binds to a common epitope on the HLA-G molecule, shown in **Figure 38** by the slight shift to the right on the x-axis of the cells transduced only with the HLA-G or β2m-HLA-G constructs, where the aHLA-A/B/C staining is higher than in the non-transduced cells. From the cells not transduced with the HLA-G constructs, it can be seen that the aHLA-G antibody is specific to HLA-G and does not recognise classical HLA Class I molecules. In order to be able to discern between the expression of HLA Class I and HLA-G, it was decided to use aHLA-A2 antibody as a representative antibody for HLA Class I. HLA-G transduced K562s were used to show that the aHLA-A2 antibody used did not also bind to HLA-G (**Figure 39**).

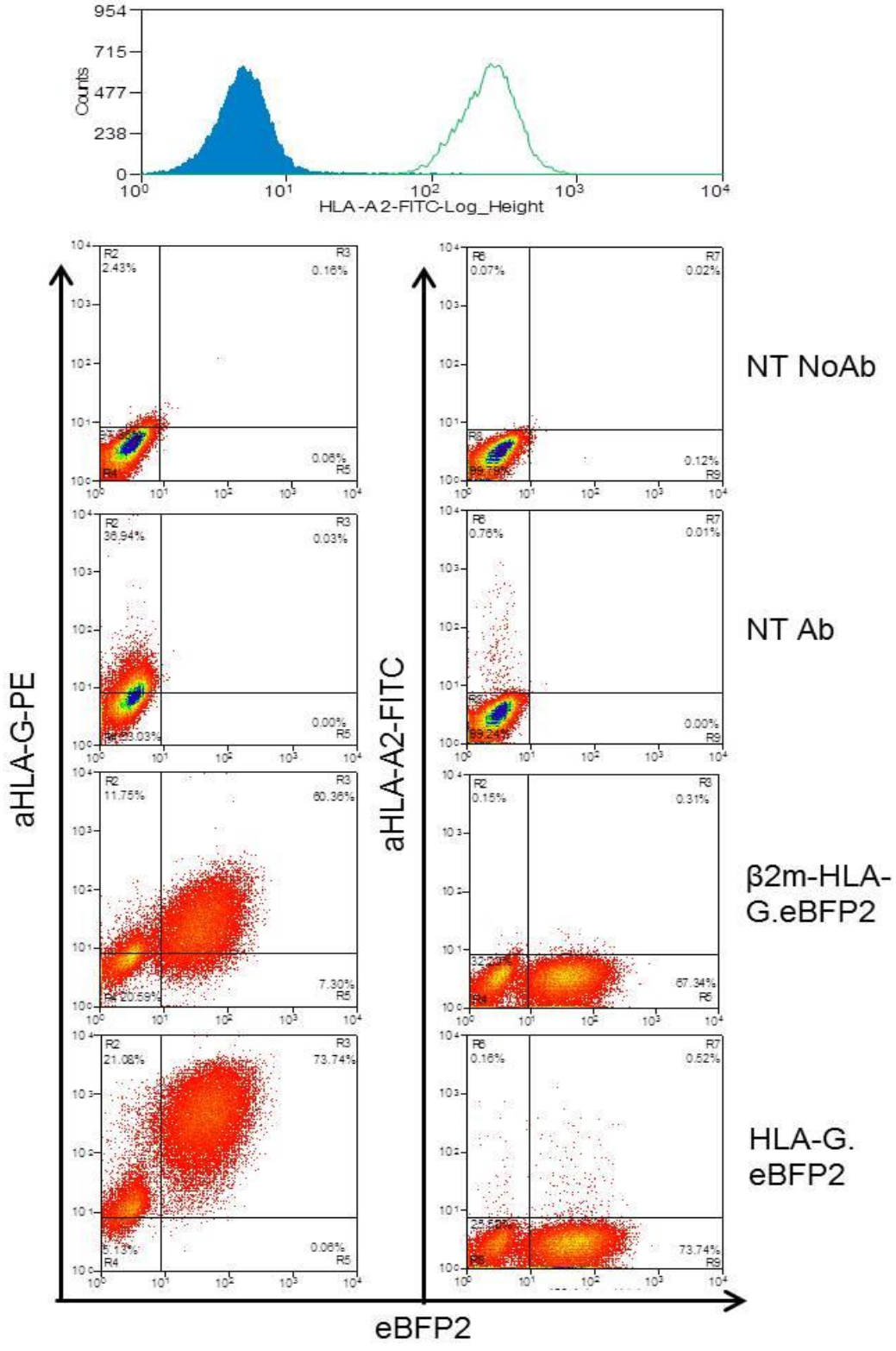

Figure 39 – Demonstrating the lack of HLA-G expression detected by aHLA-A2 antibody in HLA-G transduced K562 cells. Antibodies used were aHLA-G-PE and aHLA-A2-FITC. Both HLA-G constructs used eBFP2 as a marker gene. Top histogram shows aHLA-A2-FITC staining of PBMCs from a HLA-A2^{+} donor. NT – non-transduced; Ab – antibody. Performed once.

As this demonstrates that the HLA-G and HLA Class I molecules can now be distinguished, the experiment from **Figure 38** was repeated in donor PBMCs with the new antibody panel (aHLA-A2-FITC, aHLA-G-PE, aCD34-APC, eBFP2) (**Figure 40**).

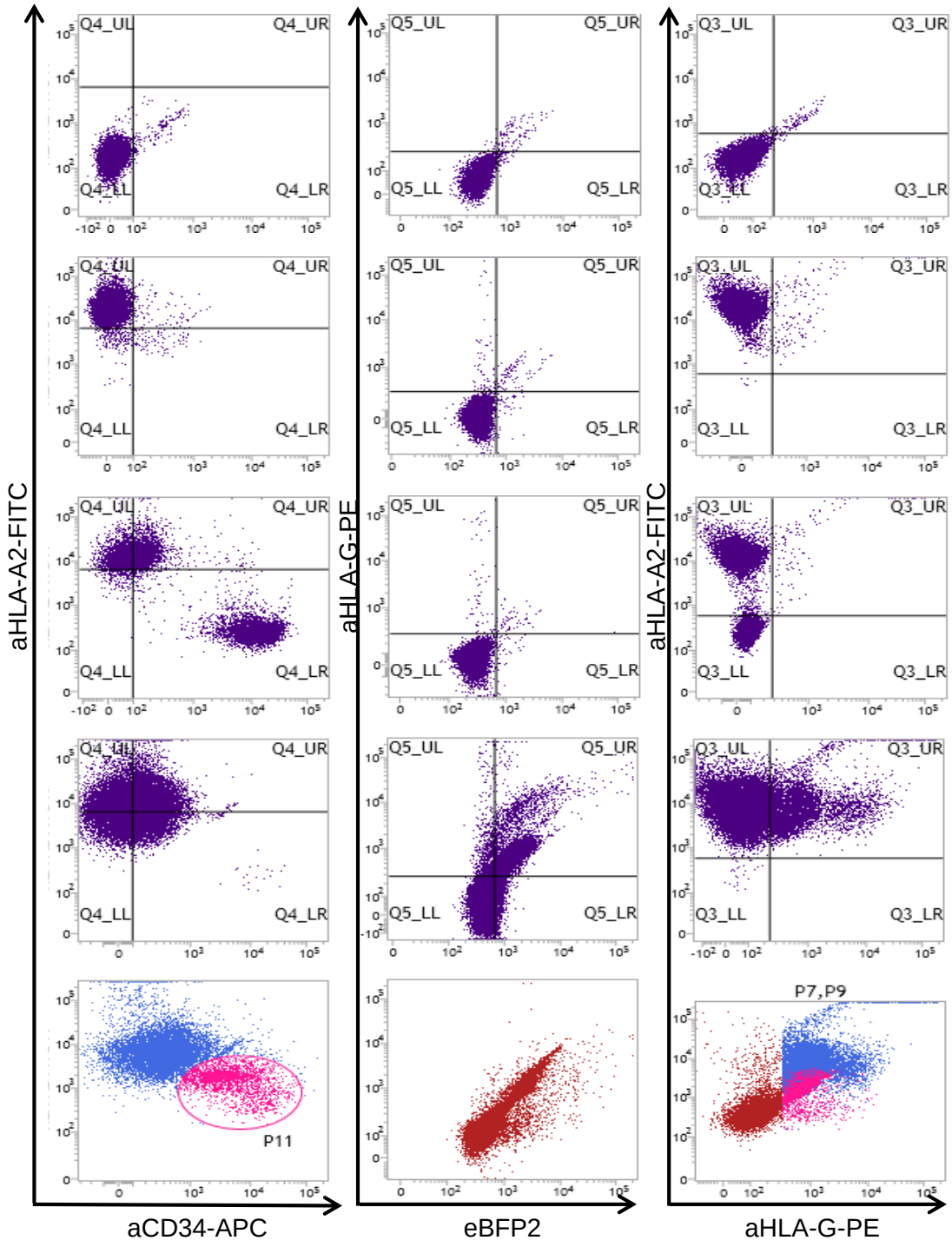

Figure 40 – HLA class I knockdown and HLA-G expression in donor PBMCs. Top row – NT – non-transduced donor PBMCs without antibody; second row – NT donor PBMCs with aHLA-G-PE and aHLA-A2-FITC antibodies; third row – donor PBMCs transduced with RQR8-2A-US11 construct alone; fourth row – donor PBMCs transduced with HLA-G.I2.eBFP2 construct alone; fifth row – donor PBMCs transduced with both constructs. Fifth row gating (gates not shown): Left dot plot shows HLA-G positive cells only. Middle dot plot shows RQR8 positive cells only. Right dot plot shows HLA-G and RQR8 positive cells in pink (P11). RQR8 expression detected with aCD34 antibody (NT – non-transduced). Performed once.

As can be seen from the bottom panel in **Figure 40**, although there is autofluorescence, it is possible to obtain cells that are both HLA-G positive and HLA Class I negative. This can be seen clearly in the bottom right dot plot, where the pink P11 gated cells are HLA-G positive, RQR8 positive and HLA-A2 negative, although this is a small proportion of the overall number of cells used in this experiment. Unfortunately, due to time constraints, it was not possible to perform replicates of this experiment, and this would be one of the key pieces of work to provide reliable data for if further work were able to be performed. If **Figure 40** is taken as an indication of co-expression, it can be said that the three strategies of HLA Class I knockdown, RQR8 expression and HLA-G expression can be combined and expressed concurrently in PBMCs. Clearly this requires further investigation, firstly to verify and quantify this and then to determine whether it would subsequently be possible to select the cells that express all of the above strategies, and how viable these cells would be after two transductions and a cell sort.

4.5 Effect of HLA-G on effector cell function

In order to begin to understand the functional capability of the HLA-G construct, a chromium release assay was carried out using PBMCs or NK cells as effector cells against K562s that were either HLA-negative (wild-type), HLA-A2-expressing or HLA-G-expressing. K562 cells had already been transduced with the SFG.HLA-A2.eGFP construct, so the generated SFG.HLA-G.eBFP2 construct was transduced into unmodified K562 cells, with the results shown below (**Figure 41**).

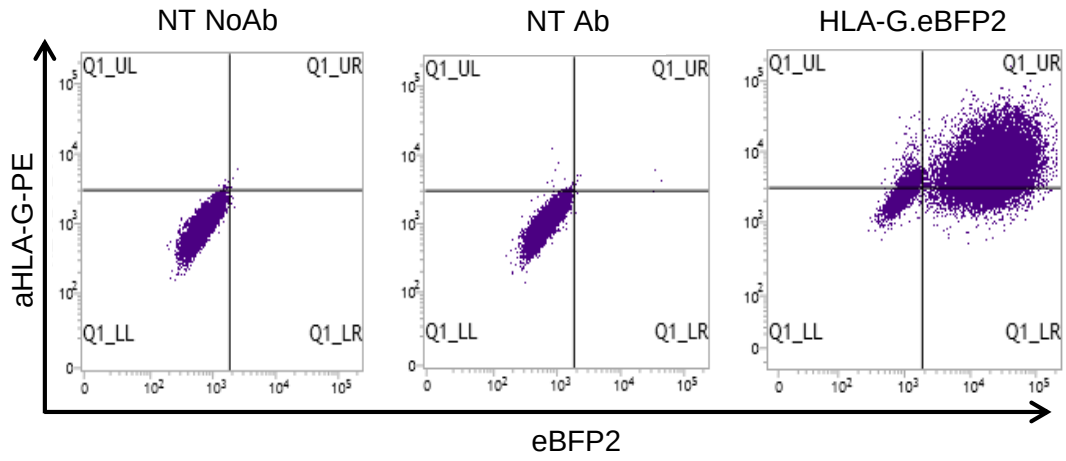

Figure 41 – HLA-G expression in K562 cells. Far right plot shows K562 cells transduced with HLA-G.I2.eBFP2 construct. NT NoAb – non-transduced K562 cells without antibody; NT Ab – non-transduced with aHLA-G-PE antibody. Performed once.

The K562 target cells were incubated with ^{51}Chromium and the release from cells was measured and quantified (**Figure 42**). From this initial assay, it is clear that NK cells are more effective at killing target cells compared to PBMCs. What is unclear is whether the HLA molecules have an effect on the activity of the effector cells. This experiment would need to be repeated with PBMCs obtained from multiple different donors in order to determine whether this is a donor-specific result or whether it is representative of all donors.

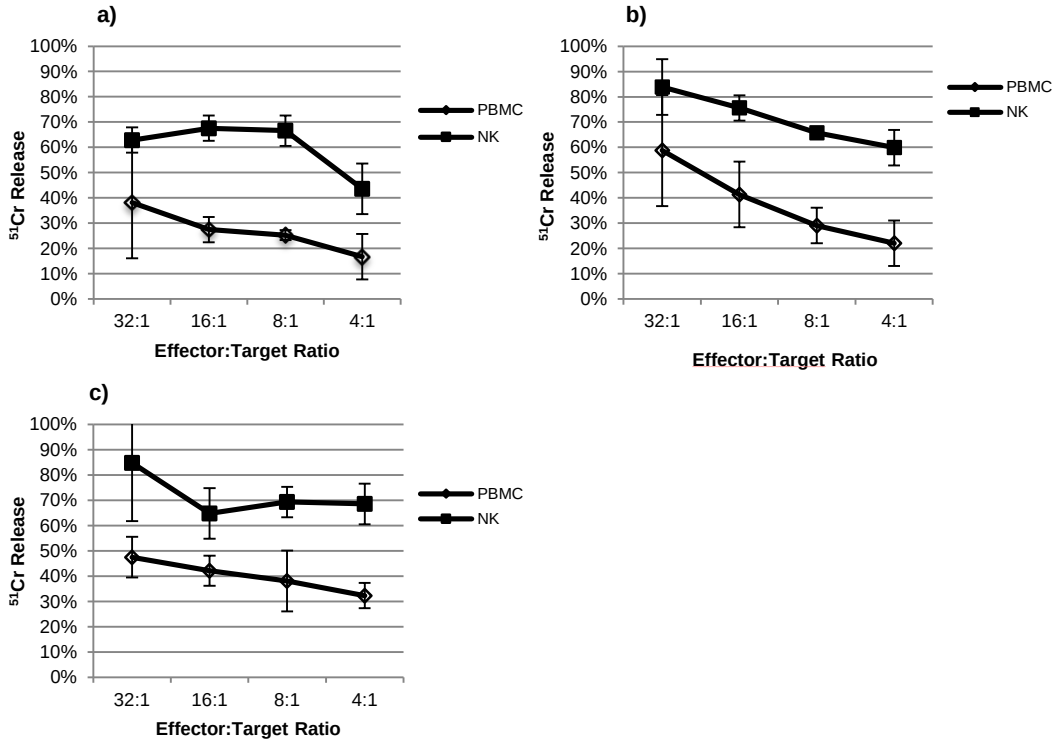

Figure 42 – Chromium release assay of PBMC/NK effector cells against K562 cell line targets expressing HLA molecules. a) NT K562 targets b) HLA-A2-expressing K562 targets c) HLA-G-expressing K562 targets. NT – non-transduced, PBMC – Peripheral blood mononuclear cells, NK – Natural-killer cells. Repeated once in duplicate.

4.6 Conclusions

In conclusion, it was possible to extract the HLA-G from genomic DNA and to clone it into an SFG vector with a marker gene. The two constructs cloned from the genomic HLA-G gene were used to transduce HLA-G in multiple cell lines, including Jurkat cells, PBMCs and SupT1s, demonstrating that it is possible to achieve detectable cell surface expression.

Since expression was better using the construct containing HLA-G on its own, without β2m attached by a linker, this was the construct that was planned to be taken forward into functional experiments. To build on the ^{51}Cr release assay (**Figure 42**), one planned experiment would be to test NK cell

proliferation in a co-culture with HLA-G expressing cells. K562 cells are an ideal cell line to use as they are HLA Class I negative and would therefore normally be targeted by NK cells (Drew et al. 1977; Saksela et al. 1979). Once a protocol and efficacy is demonstrated using K562 cells, PBMCs would then be investigated. The various subsets of cells within the PBMC population would be interrogated to determine whether any of these have an impact on the functionality of NK cells. The impact of transduction on expression of NK ligands would also need to be examined, as if NKG2D ligands are increased as a result of transduction, this might overcome the effect of HLA-G.

HLA-G was expressed in cells alongside RQR8 and US11, resulting in the discovery that the aHLA-A/B/C Class I antibody being used also recognised a common epitope on HLA-G. Despite the lack of functional data, it appears possible to co-express RQR8, US11 and HLA-G in donor PBMCs, meaning that this has the potential to be the platform on which the universal T cell could be built if these strategies were proven to be functionally effective.

4.6 Final Conclusions

- Two HLA-G constructs have been successfully cloned from genomic DNA and repaired where necessary.
- Both HLA-G constructs have been shown to be expressed in Jurkat, K562 and SupT1 cell lines, as well as in PBMCs, with the construct containing HLA-G alone expressing more highly than the construct also containing β2m attached with a linker.
- When attempting to express the HLA Class I knockdown constructs with the HLA-G constructs, the aHLA-A/B/C antibody was shown to also bind to an epitope on HLA-G, resulting in a need to alter the antibody panel being used.
- The RQR8-2A-US11 construct and HLA-G construct can be co-expressed in PBMCs, although only in a small proportion. This also

needs further verification and quantification in order to make a reliable assessment.

- K562 cells expressing HLA-A2 or HLA-G were killed by PBMCs and NK cells but it was not possible to determine whether there was any difference between the cell lines. It was determined that NK cells had a higher capability to kill target cells compared to PBMCs.

Chapter Five: TCR knockdown

5.0 Aims

- To prevent or reduce effects of GvHD as a result of TCR signalling.
- To investigate methods of TCR knockdown from the T cell surface.
- To verify success of these methods of knockdown.

5.1 Introduction

TCRs are widely expressed on the cell surface of T cells. Their role is in antigen recognition of antigen presented to them by APCs, which then results in either anergy or activation depending on the immunogenicity of the antigen presented (Janeway Jr et al. 2001). If allogeneic T cells are used in therapy, then the TCRs expressed by the therapeutic cells can activate the CTL response when they encounter antigens from the recipient's tissues, leading to GvHD. In adoptive immunotherapy, transfer of antigen-specific T cells is used in an attempt to minimise GvHD. TCR knockdown is a mechanism which might be useful in preventing the GvHD response. If a CAR is introduced, this would have the effect of promoting the GvL effect, and also prolonging the persistence and survival of adoptively transferred therapeutic T cells *in vivo*.

5.2 Approaches used for TCR knockdown

There are multiple methods that can be used to knock down expression of proteins from a cell. They can be targeted at the genomic level by methods such as ZFNs (discussed in chapter four); at the mRNA level by siRNA or miRNA (tested and discussed in chapter three); at protein level by retention sequences and intrabodies (Pelham 1990).

As used previously in an attempt to achieve HLA Class I knockdown, one strategy that was used in this chapter was to combine the specificity of an antibody against a protein with a retention sequence to retain the protein within

the cell and prevent its surface expression. Retention sequences were characterised by Munro and Pelham (1986), who showed that an amino acid sequence of KDEL which was present on the C-terminus of three luminal ER proteins was responsible for their retention in the ER. It was subsequently shown that this sequence, could be used to retain proteins in the ER and other locations (Pelham 1990; Andres et al. 1990). To target a protein in a specific manner and retain it within the ER, the strategy in this study required a molecule attached to a retention sequence that would bind to the protein of interest. This approach allows for a specific targeting of a protein and for intracellular retention, but the drawback is that it might cause ER stress due to the large amount of protein being retained in the ER. This could cause the activation of the UPR and would lead to up-regulation of ER stress proteins including IRE1, ATF6 and PERK, leading to ERAD, translational attenuation and possible apoptosis (Rutkowski & Kaufman 2004; Schröder & Kaufman 2005). Other ER retention sequences that were tested to obtain TCR knockdown were the 'KKAA' retention sequence and the E19 adenovirus protein sequence 'KYKSRRSFIDEKKMP' (Pelham 2000).

Another similar strategy was tried, using the 'SEKDEL' ER retention sequence attached to a bacterial 'superantigen' to knockdown the TCR. Bacterial 'superantigens' are a family of 40 protein toxins produced by *Staphylococcus aureus* and *Streptococcus pyogenes* that bind to the TCR and MHC class II in order to cause mass T cell stimulation, resulting in toxic shock syndrome (Fraser & Proft 2008; Proft et al. 2000). A relatively small amount of superantigen in a human can cause a sudden rise in cytokine levels, including IL-2, IFN-γ and TNFα (Jupin et al. 1988). However, at lower levels, during the early stages of infection, the effect of the superantigen is to induce a localised immunosuppressive effect in order to prevent clearance of the bacterium. Studies have shown that the superantigens have the ability to bind directly to MHC Class II and the TCR-Vβ chain outside of the normal paratope for peptide-MHC recognition (Scholl et al. 1989; Dellabona et al. 1990). There are numerous superantigens that have been described, with SMEZ-2 (*S.pyogenes*) having a high potency and therefore likely to have the highest affinity for the TCR-Vβ (4.1 and 8.1) and MHC Class II. This was cloned with

a signal sequence and attached to the ER retention sequence ‘SEKDEL’ before being tested in donor PBMCs.

The final strategy used to obtain TCR knockdown was TALENs. TALENs are a fusion of a transcription activator-like effector, derived from the plant pathogen *Xanthomonas spp.*, with the FokI nuclease. They are genome-editing molecules that are designed to induce a double strand break (DSB) in the targeted area of the genome. TALENs recognise a sequence of DNA through a series of repeated variable di-residues (RVDs). Each repeat is 33-35 amino acids long, with two hypervariable amino acids at the 3’ end which dictate which DNA base is targeted (Christian et al. 2010; Li et al. 2012; Hockemeyer et al. 2011). Combinations of these repeats can be joined together to target a DNA sequence of 18-20bp. Attached to the end of this is the catalytic domain of the FokI nuclease. FokI requires dimerization for catalytic activity, so two TALENs are used, one binding to the ‘left’ of the target sequence, the other binds to the ‘right’ end of the target sequence (Miller et al. 2011).

Knockdown of the TCR by using a protein targeting method was preferable to using DNA editing molecules as it removed the risk of mutations caused by NHEJ, making this project safer and more viable. It also allows the use of a viral packaging and delivery system and therefore can be combined with other strategies, reducing the number of selection stages to obtain a homogenous cell product.

5.3 Testing of constructs

After constructs were cloned, testing was performed in multiple cell lines, including TCRαβ-negative SupT1 cells, Jurkat cells and TCRko Jurkat cells as well as donor PBMCs. TCRαβ-negative SupT1 cells were used as they could be engineered to express a TCR, thereby creating an engineered cell line that had an ideal negative control for expression tests. SupT1s (negative for TCR expression) were transduced with a codon optimised TCR specific for the

mHag HA1 (den Haan et al. 1998) (**Figure 43**). TCR negative Jurkat cells (patient-derived cell line lacking TCR α and β chains) were also obtained to be used as a negative control cell line (kindly provided by Hans Stauss, Royal Free) (**Figure 44**). It was these engineered cell lines that were initially used for testing the aTCR antibody constructs with the ER retention sequences attached.

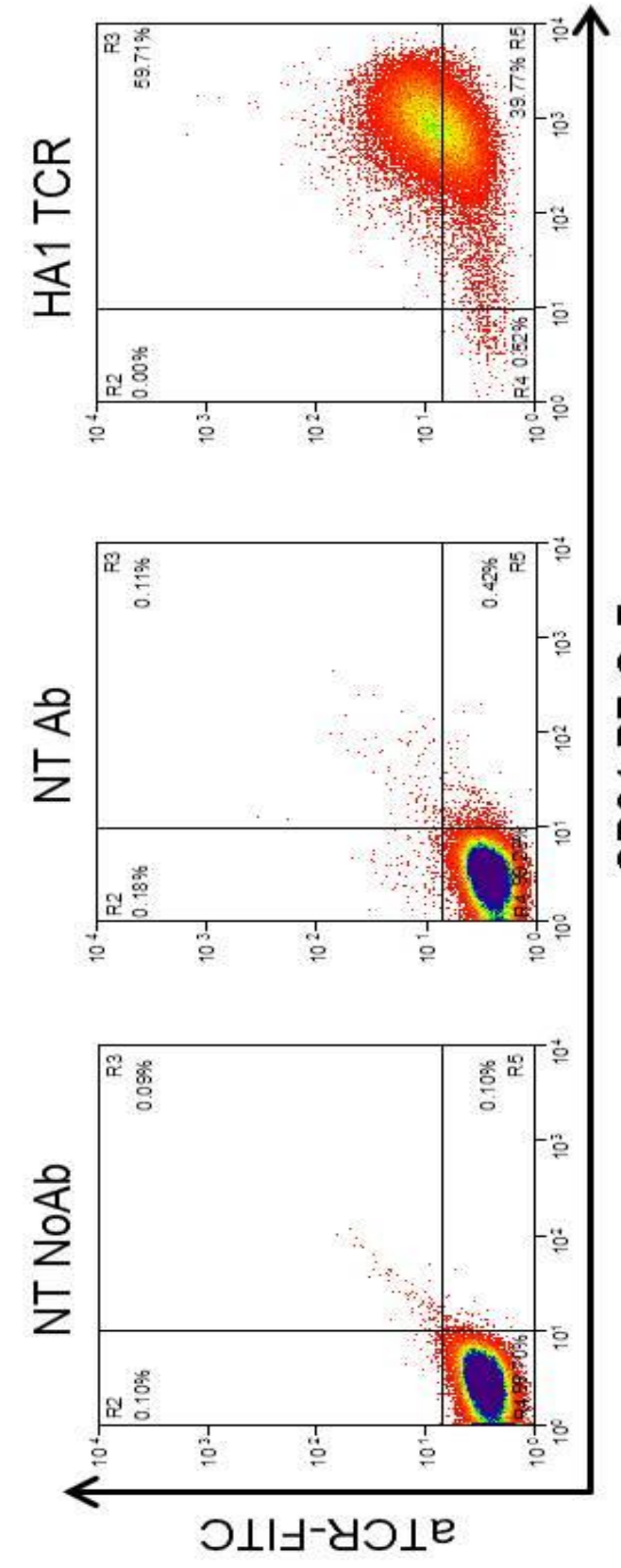

Figure 43 – HA1-TCR expression in SupT1 cells. A construct containing the HA1-TCR and truncated CD34 as a marker gene was transduced into SupT1s. NT – non-transduced cells; Ab – antibody. Repeated once.

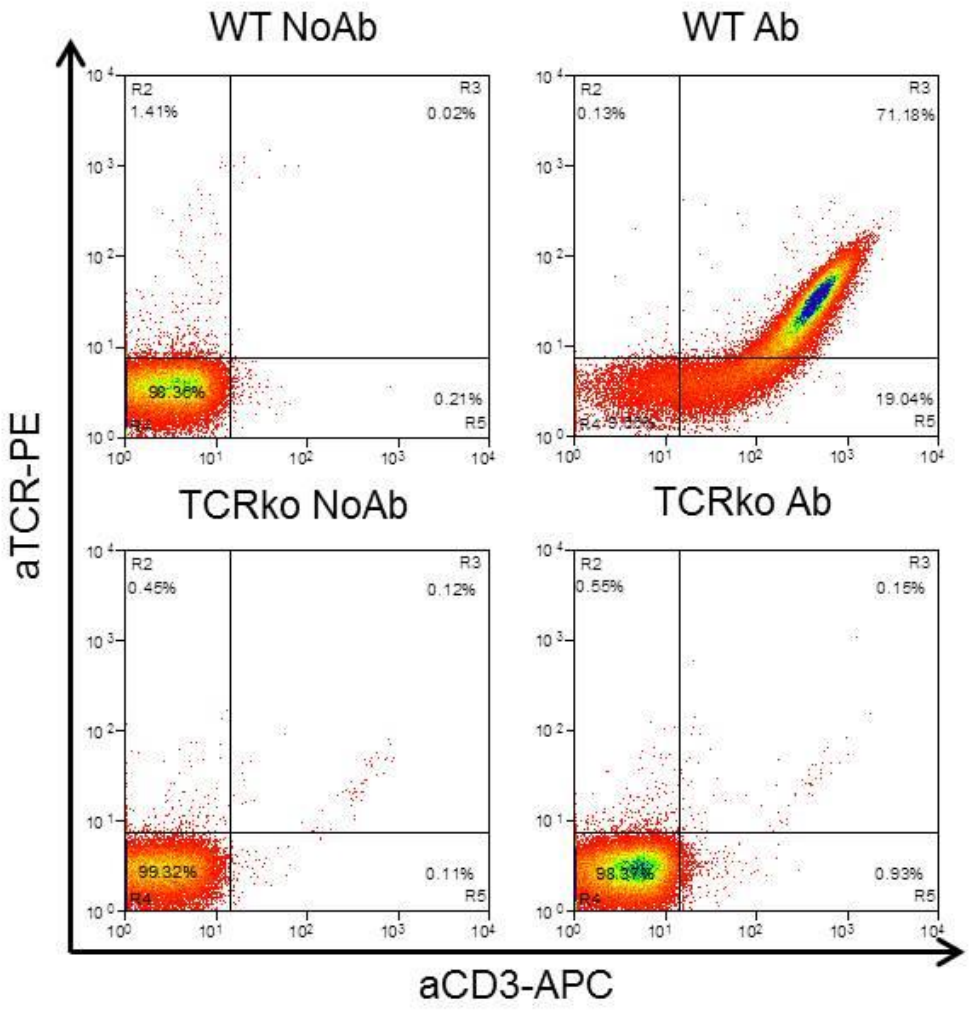

Figure 44 – TCR expression on wild-type (WT) and TCR negative (TCRko) Jurkat cells. Cells were stained with aTCR-PE antibody (Ab) and aCD3-APC antibody. Performed once.

5.4 Attempting to knockdown the TCR using aTCR antibodies attached to ER retention sequences

5.4.1 Testing that aTCR antibodies bind to target

The first strategy used for TCR knockdown was to attach retention sequences to antibodies which have a high affinity for the TCR and its surrounding components. Single chains of OKT3, BMA031 and truncated pre-T cell

receptor alpha (dPTCRA) with the ‘SEKDEL’ retention sequence had previously been cloned (**Figure 45**). The ‘SEKDEL’ ER retention sequence is one that has been demonstrated to achieve effective retention of target proteins in the ER (Pelham 1990). OKT3 and BMA031 are both antibodies that have been generated against the TCR-CD3 complex (Borst et al. 1990; Midtvedt et al. 2003), whilst PTCRA is involved in the development of immature αβ T cells (Fehling et al. 1995). A truncated form of PTCRA might bind to the TCR during assembly and prevent surface expression. To test that the OKT3 and BMA031 antibodies bound to the TCR, scFvs attached to a human Fc were generated in secreted format and transfected into 293T cells. Retroviral supernatants were harvested before being incubated with Jurkat cells, followed by the addition of anti-human-Fc antibody to confirm association of the secreted scFv to the Jurkat cells. An aGD2 scFv construct was used as a negative control (**Figure 46**).

BMA031 construct:

*METDTLLLWVLLLWVPGSTG*EVQLQQSGPELVKPGASVKMSCKASGYKFTSYVMH
WVKQKPGQGLEWIGYINPYNDVTKYNEKFKGKATLTSDKSSSTAYMELSSLTSED
SAVHYCARGSYYDYDGFVYWGQGTLVTVSASGGGGSGGGGSGGGGSQIVLTQSPA
IMSASPGEKVTMTCSATSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPARFSGS
GSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKRSDP**SEKDEL**

OKT3 construct:

*METDTLLLWVLLLWVPGSTG*QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMH
WVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSED
SAVYYCARYYDDHYCLDYWGQGTTLTVSSSGGGGSGGGGSGGGGSQIVLTQSPAI
MSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAHFRGSG
SGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRSDP**SEKDEL**

PTCRA construct:

*MAGTWLLLLLALGCPALPTGVG*GTPFPSLAPPIMLLVDGKQQMVVVCLVLDVAPP
GLDSPIWFSAGNGSALDAFTYGPSPATDGTWTNLAHLSLPSEELASWEPLVCHTG
PGAEGHSRSTQPMHLSGEASTARTCPQEPLRGTPGGALWLGVLRLLLFKLLLFDL
LLTCSCLCDPAGPLPSPATTTRLRALGSHRLHPATETGGREATSSPRPQPRDRRW
GDTPPGRKPGSPVWGEGSYLSSYPTCPAQAWCSRSALRAPSSSLGAFFAGDLPPP
LQAGAARSDP**SEKDEL**

Figure 45 – Design and sequences of constructs targeted at TCR knockdown by ER retention. Top two constructs show amino acid sequence – signal peptide is in italics, heavy chain is highlighted yellow and light chain is highlighted in green. ‘SEKDEL’ ER retention sequence is highlighted in blue. Bottom construct shows amino acid sequence of the PTCRA construct. Signal peptide is in italics, protein sequence is highlighted in grey.

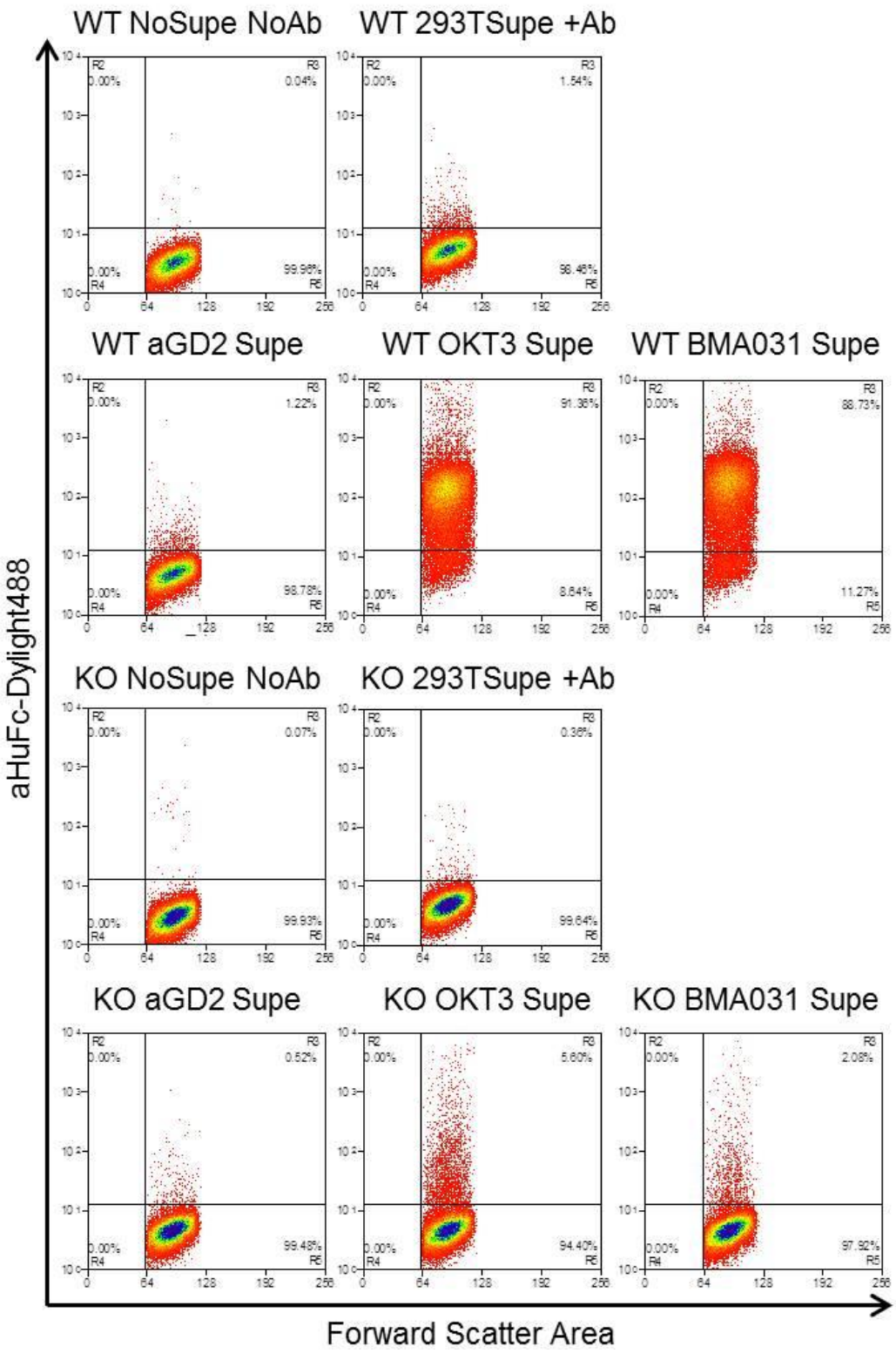

Figure 46 – Testing of anti-TCR antibody apecificity for the TCR. Supernatants containing secreted Abs were generated by transfection of 293Ts with the relevant constructs. Supernatants were used to stain cells, followed by anti-Human-Fc detection as follows: Rows one and two – wild-type Jurkat cells stained with secreted Abs; Rows three and four – TCRko Jurkat cells stained with secreted Abs. Ab – antibody; 293T – supernatant from NT 293Ts used followed by secondary aHuman-Fc-Dylight 488 antibody. Performed once.

5.4.2 Knockdown of TCR by aTCR-SEKDEL constructs in Jurkat cells

TCR knockdown was first tested by transducing wild-type Jurkats with retroviral supernatant containing the various ‘SEKDEL’ constructs: native TCR (nTCR), aHuman-CTLA4-SEKDEL, OKT3-SEKDEL, BMA031-SEKDEL and dPTCRA-SEKDEL constructs. TCR knockout Jurkats (**Figure 47**). The construct that targeted CTLA-4 was used as it is expressed on the surface of T cells and acts as a control in this experiment.

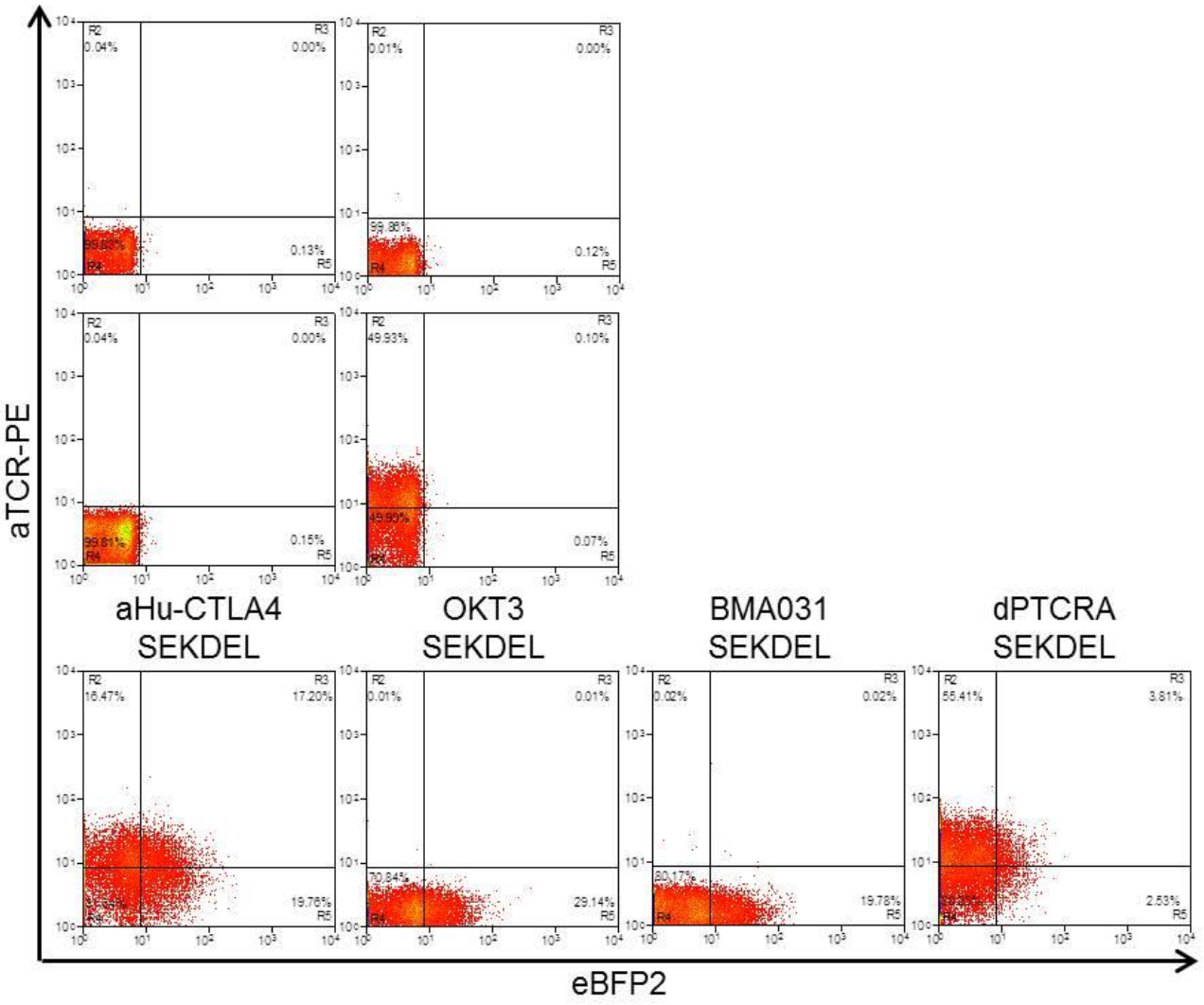

Figure 47 - aTCR-SEKDEL constructs tested in Jurkat cells. Top row shows NT TCRko Jurkat cells (-/+ Ab) and second row shows NT wild-type Jurkats (-/+ Ab). Third row shows WT Jurkats transduced with the relevant constructs and stained with aTCR-PE antibody. The marker gene for the constructs was eBFP2. NT – non-transduced; Ab – Antibody. Repeated once.

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As can be seen, this strategy looks to have been successful in knocking down the TCR expressed on wild-type Jurkat cells as a result of transduction with either the OKT3-SEKDEL or BMA031-SEKDEL construct. The dPTCRA-SEKDEL construct failed to work, but will be carried forward as a negative control construct.

5.4.3 Knockdown of TCR by aTCR-SEKDEL constructs in donor PBMCs

Since two of the constructs appeared to be successful in knockdown of the TCR from Jurkat cells, the next rational step was to test the constructs in donor PBMCs (**Figure 48**).

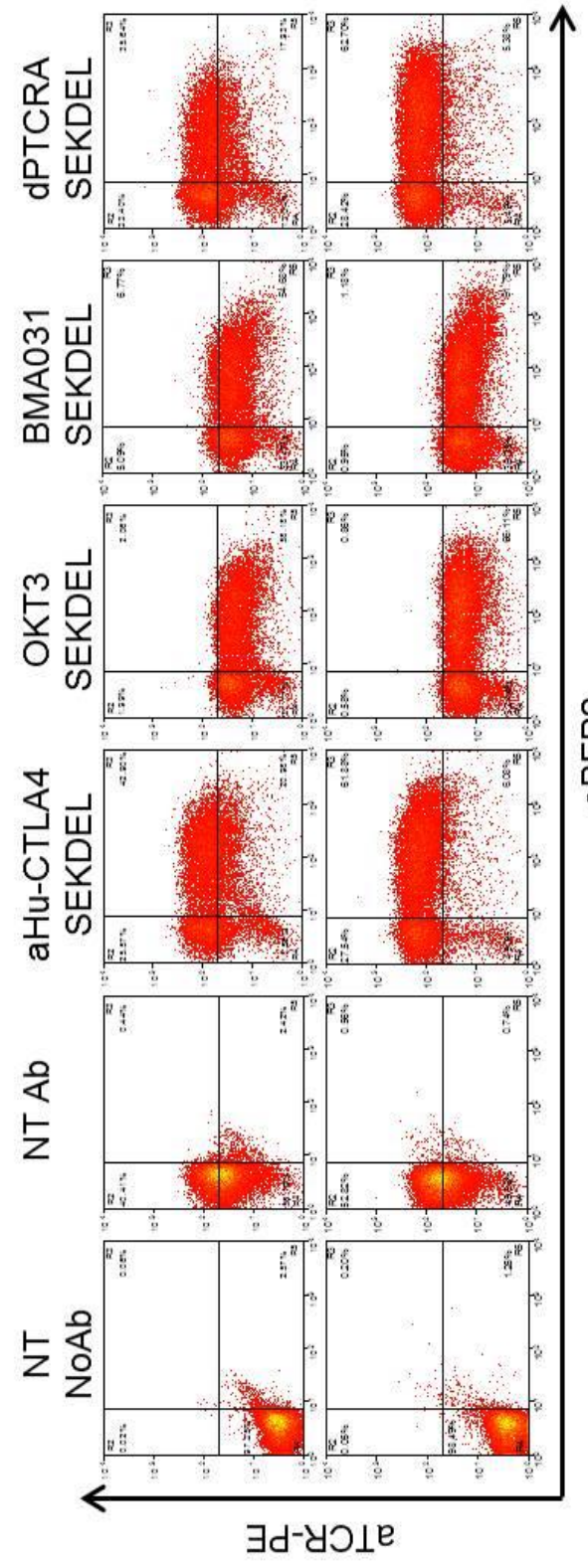

Figure 48 – aTCR-SEKDEL constructs tested in donor PBMCs. Cells were transduced with retroviral supernatant containing the relevant constructs. The marker gene used was eBFP2. aHuCTLA4-SEKDEL and dPTCRA-SEKDEL were used as negative controls. Cells were analysed by FACS using aTCR-PE antibody and marker gene expression. NT – non-transduced; Ab – antibody. Repeated twice, figure shows representative dot plots for two donors.

From this data, there initially looked to have been successful, although partial, TCR knockdown by the OKT3-SEKDEL and BMA031-SEKDEL constructs. Unusually though, the non-transduced cells also appeared to have lost some of their TCR expression despite being eBFP2 negative. It might also be useful to quantify the level of TCR expression on the cell surface for future experiments.

5.4.4 Investigation into TCR knockdown

5.4.4.1 Cell line investigation

In order to elucidate what was occurring to cause these unusual results, further investigation was required. Firstly, the aim was to discover why there was only partial knockdown of the TCR. To do this, the HA1-TCR SupT1 cell line (**Figure 43**) was transduced with the aTCR-SEKDEL constructs to determine if there was a Golgi saturation event due to the expression of the native TCR being higher than the transgene expression (**Figure 49**).

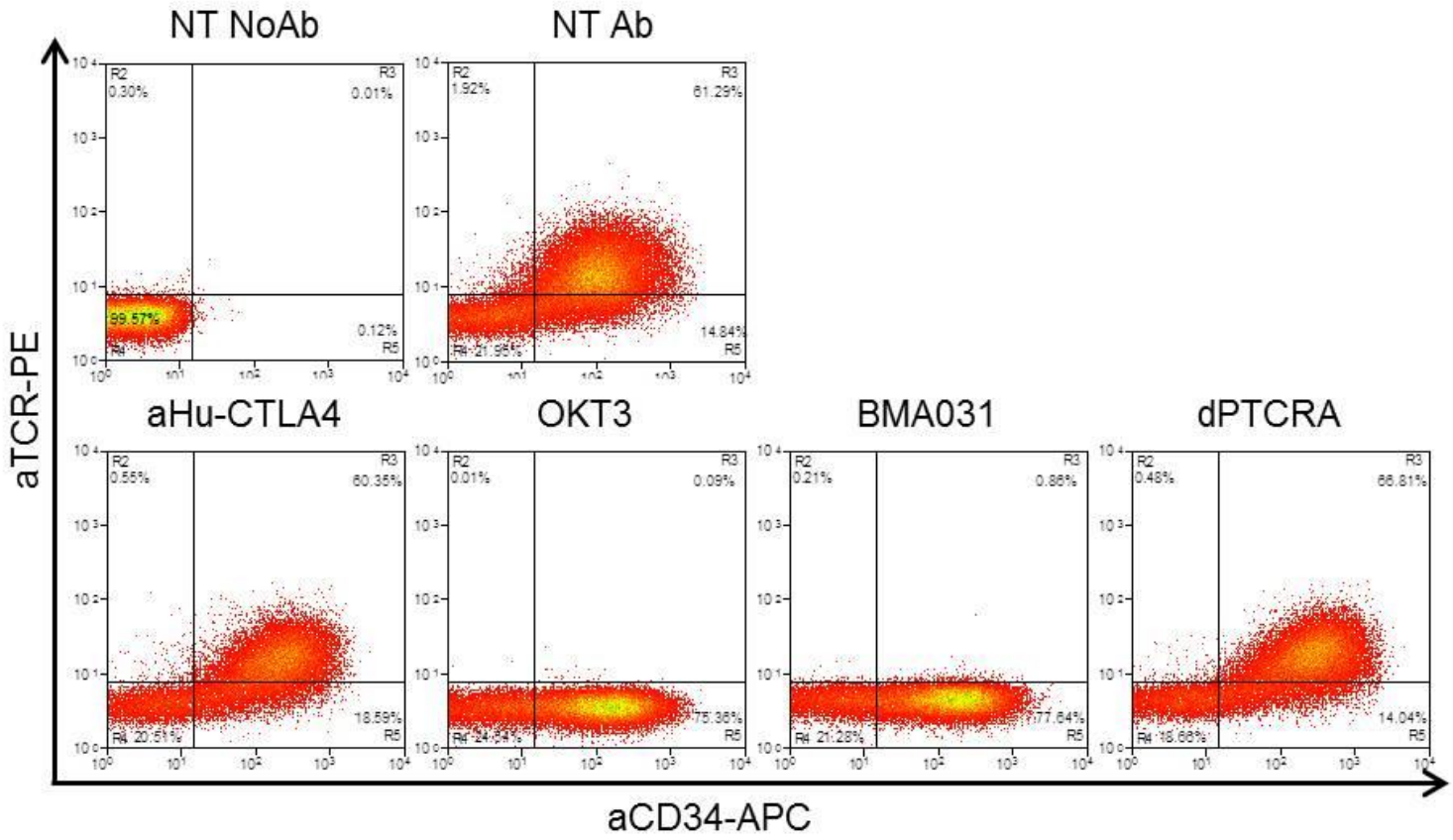

Figure 49 – Transduction of aTCR-SEKDEL constructs into aHA1-TCR-SupT1 cells. Top row shows NT aHA1-TCR SupT1 cells, bottom row shows aHA1-TCR SupT1 cells transduced with the relevant aTCR-SEKDEL constructs. Cells were stained with aTCR-PE and aCD34-APC antibodies. NT – non-transduced. Ab – antibody. Performed once.

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Here, the OKT3-SEKDEL and BMA031-SEKDEL constructs clearly work effectively in knocking down the HA1-TCR from the cell surface. This suggests that the knockdown constructs are limited in their efficiency by their expression, leading to the conclusion that the levels of TCR expression on the T cell surface are too high to be completely removed as the transgene is unable to effectively retain all of the TCRs within the ER and Golgi. To verify this further and determine if Golgi saturation was occurring, with more protein being expressed than can be retained by the construct, donor PBMCs and SupT1s were transduced with a construct that contained a truncated CD33 molecule along with the eGFP marker gene. Following this, those populations were then transduced with retroviral supernatant containing an aCD33-SEKDEL.eBFP2 construct (**Figure 50**).

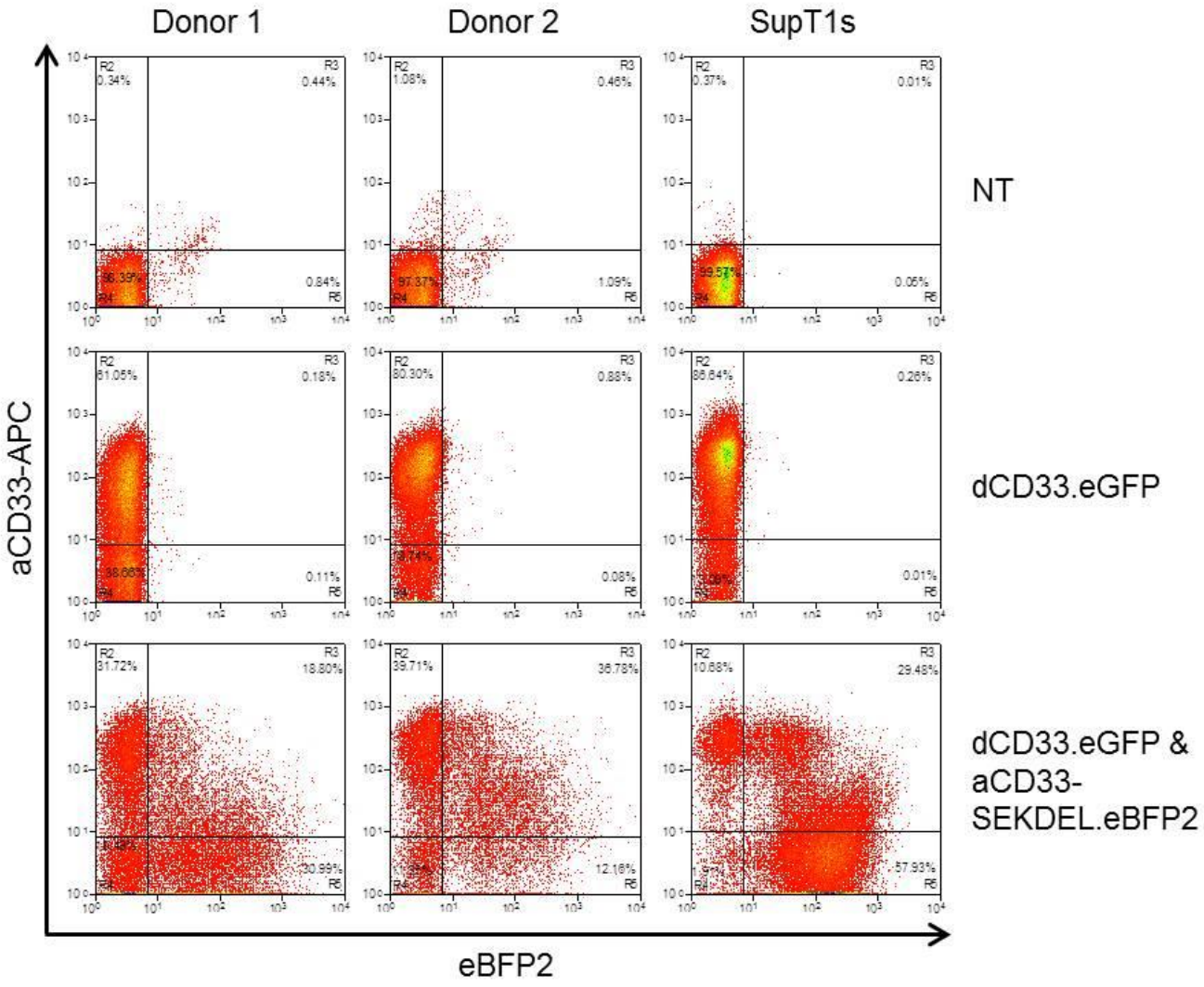

Figure 50 – dCD33 expression and knockdown in donor PBMCs and SupT1 cells. Cells were transduced with dCD33.eGFP construct (second row) before being subsequently transduced by an aCD33-SEKDEL.eBFP2 construct. Cells were analysed by FACS using aCD33-APC antibody and marker gene expression. NT – non-transduced.

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The average aCD33-APC MFI for the dCD33 transduced (eGFP-gated) PBMCs was 285.03 (S.D. 53.24), and the aCD33-APC MFI for the double-transduced populations (both eBFP2 and eGFP positive) was an average of 100.33 (S.D. 47.33), a reduction of 65%. In the SupT1s, there was a reduction in aCD33-APC MFI from 291.10 to 43.25, a drop of 85%. This pattern replicated what had been seen previously, indicating that Golgi saturation was not occurring as both the target and the effector genes were both transgenes and therefore would have been expressed at a ratio of around 1:1. It can be concluded therefore, that the partial knockdown is very likely to be as a result of levels of TCR expression being higher than transgene expression.

5.4.4.2 Co-cultures

Another aspect of the original FACS analysis from **Figure 48** was that the NT cells also had a reduced level of TCR surface staining. To try to resolve this, PBMCs were transduced with the SEKDEL constructs and then added to NT PBMCs at a ratio of 1:1 and incubated together for 48hrs (**Figure 51**).

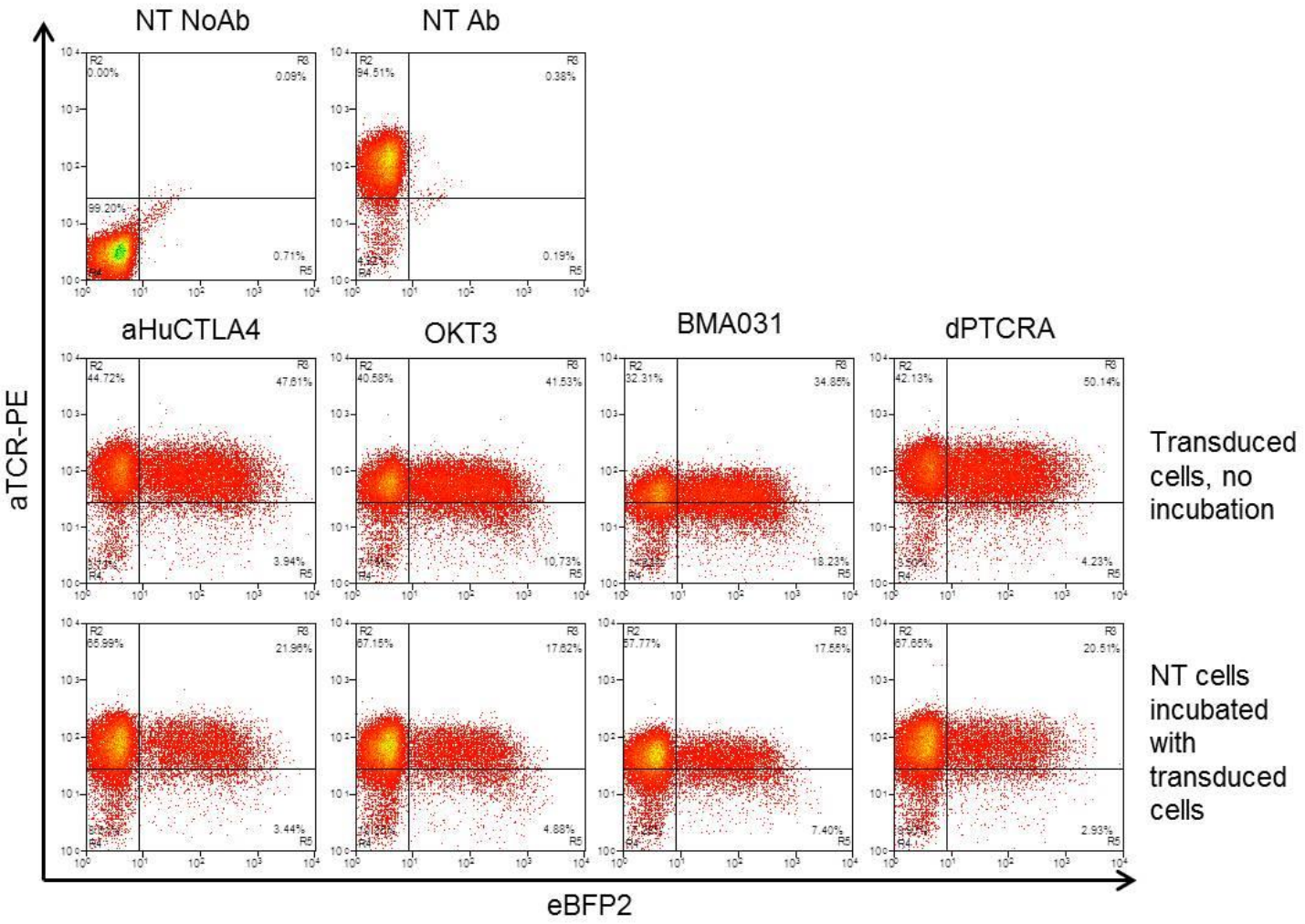

Figure 51 - 1:1 48hr incubation (autologous setting) of NT donor PBMCs with donor PBMCs transduced with aTCR-SEKDEL constructs (second and third rows). Cells were stained with aTCR-PE antibody, and the marker gene for the aTCR-SEKDEL constructs was eBFP2. NT – non-transduced; Ab – antibody. Performed once.

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In this experiment, if the construct was being secreted, it would have been expected to see three populations – one of NT PBMCs that had normal levels of aTCR staining, one of NT PBMCs with reduced levels of aTCR staining and one of transduced PBMCs also with reduced levels of aTCR staining. As the NT PBMCs that were introduced also had reduced levels of aTCR staining after 48hrs incubation, this suggests that there is a cell to cell contact mechanism occurring, with several possible theories that could explain the results obtained. One theory is that the SEKDEL construct might be binding to the TCR, but not causing retention, leaving the construct bound to the TCR on the cell surface, possibly blocking antibody binding (or vice versa) and leading to the reduction in TCR staining seen previously. It is also possible that the TCRs on the NT PBMCs are binding to the SEKDEL construct bound to the surface of the transduced PBMCs, again, blocking antibody binding and leading to the reduction in staining. Another explanation for the reduction in staining seen on NT PBMCs is that the SEKDEL constructs might be secreted and that is the mechanism by which they are able to bind to the NT PBMCs and block antibody binding. Fluorescence microscopy might be one technique that would help in investigating these hypotheses.

To further investigate whether the SEKDEL constructs were being secreted or whether there was a cell to cell contact process occurring, supernatant from the transduced PBMCs was used to stain NT PBMCs, followed by TCR-PE antibody (**Figure 52**). It has already been shown that the OKT3 and BMA031 antibodies bind to the TCR (**Figure 46**) and, with comparison to **Figure 52**, this shows that there was no reduction in staining, leading to the conclusion that the constructs are not being secreted.

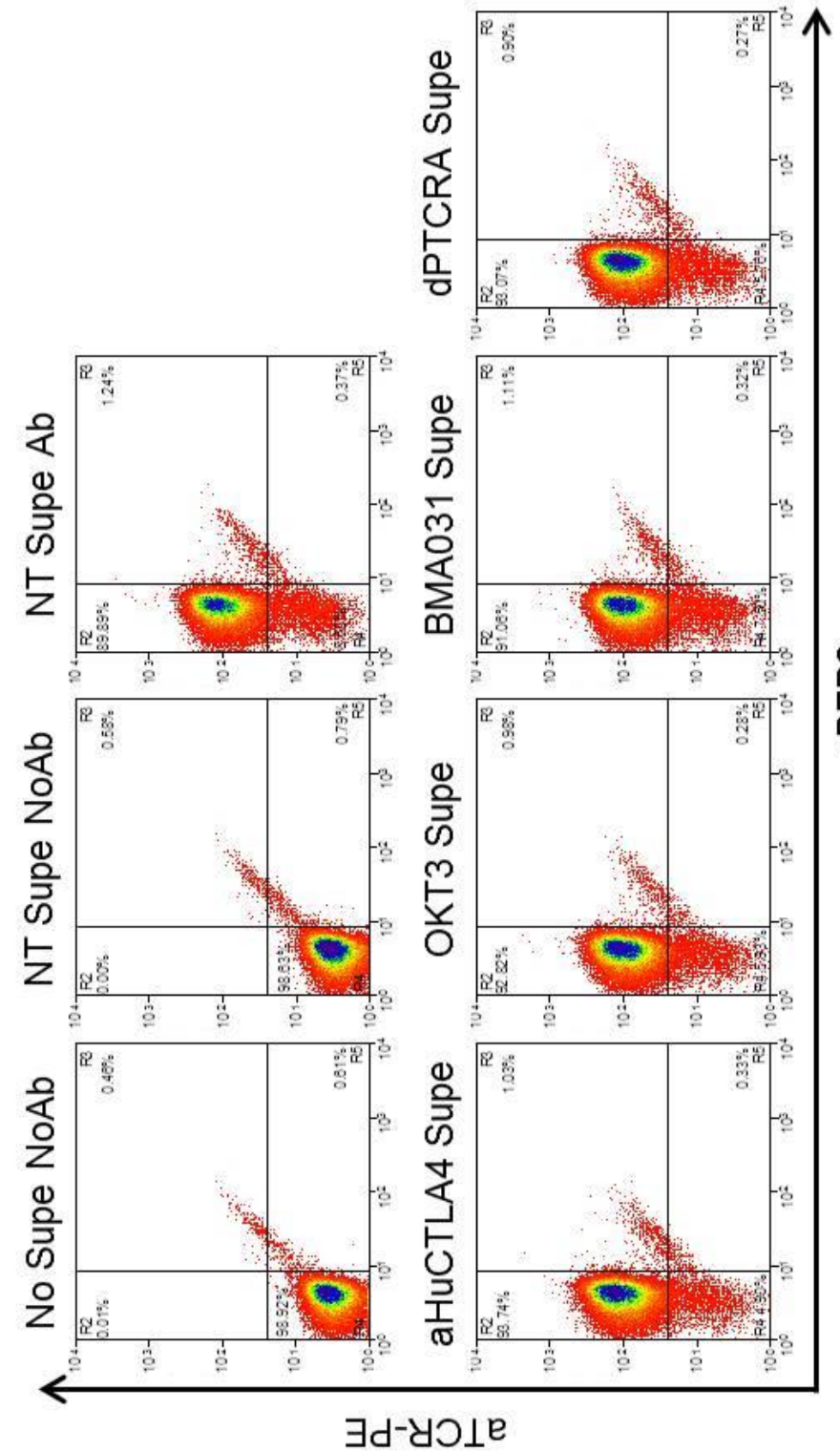


Figure 52 - NT donor PBMCs stained with supernatant from PBMCs transduced with aTCR-SEKDEL constructs, followed by staining with aTCR-PE antibody. NT – non-transduced; Ab – antibody. Performed once.

To further prove this, NT PBMCs and transduced PBMCs were incubated 1:1 in transwells, preventing cell to cell contact, but allowing exchange of anything that might be secreted (**Figure 53**). Again, this showed that there was no reduction in TCR staining on the NT PBMCs, supporting the theory that the constructs are bound to the TCR expressed on the cell surface and are blocking antibody binding on the transduced and adjacent NT cells. This may suggest a novel mechanism for achieving cell surface receptor or molecule blocking.

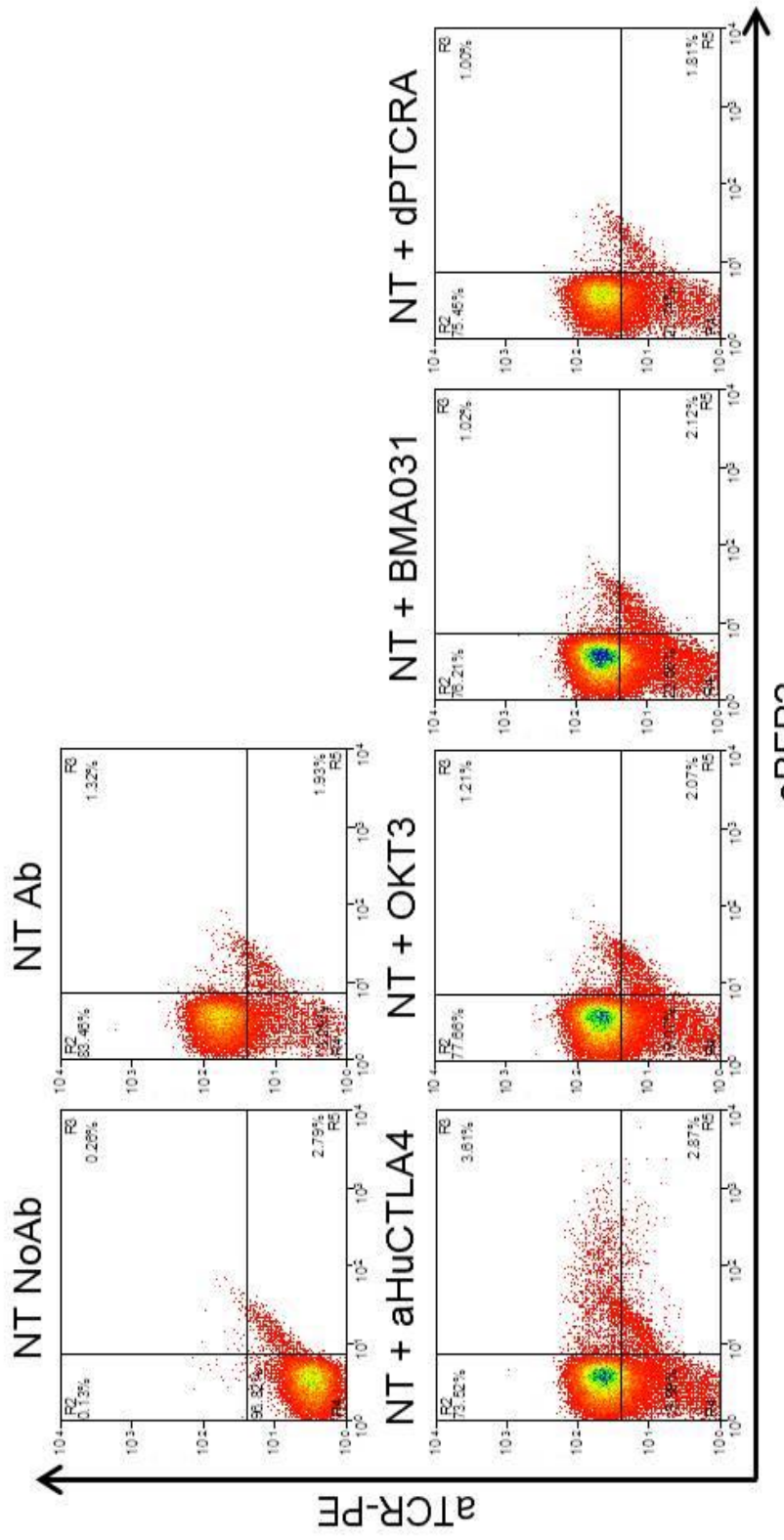


Figure 53 – 48hr transwell 1:1 incubation of NT PBMCs with autologous aTCR-SEKDEL transduced PBMCs. NT – non-transduced; Ab – antibody. Performed once.

5.4.5 Knockdown of TCR by aCD3 ER retention constructs

Since the strategy of targeting the TCR complex for knockdown caused unwanted effects, including blocking the TCR of neighbouring T cells, it was decided that if the TCR could not be retained in the ER directly, then it might be possible to retain the TCR in the ER by retaining the CD3 molecule. CD3 has multiple dimeric signalling domains, CD3δε, CD3γε and CD3ζζ (**Figure 66**). These non-covalently associate with the TCR as a result of specific residue interactions with one basic residue and a pair of acidic residues in the transmembrane domains of the CD3 components (Call & Wucherpfennig 2007). Each of the subunits of the TCR-CD3 complex have ER retention or retrieval signals, and regulation of each of these is key to obtaining cell surface expression. CD3γ has been shown to have a retention signal, but CD3γε dimers have been shown to express on the cell surface after deletion of the CD3ε ER retention signal, suggesting that the ε signal is dominant (Delgado & Alarcón 2005). Therefore, if it is possible to increase the ER retention of one or multiple CD3 components, this could lead to TCR retention also. To achieve TCR retention in the ER, one strategy was to replace the endodomains from the CD3 subunits with the 'KYKSRRSFIDEKKMP' peptide from the adenovirus E19 protein (Pelham 2000) (**Figure 54** and **Figure 55**). The risk with using this as a retention signal is that the long endodomains of neighbouring CD3 components might prevent access to the coat protein I (COPI) complex coat proteins, although retention with COPI proteins has been suggested to occur during TCR assembly as a result of the CD3ε subunit (Mallabiabarrena et al. 1995). Another strategy was to clone the following amino acid sequence, comprising of a serine-glycine linker attached to the 'KKAA' ER retention sequence, resulting in the peptide 'SGGGSKKAA' being added to the full-length endodomain of each of the CD3 components (Pelham 2000) (**Figure 56**).

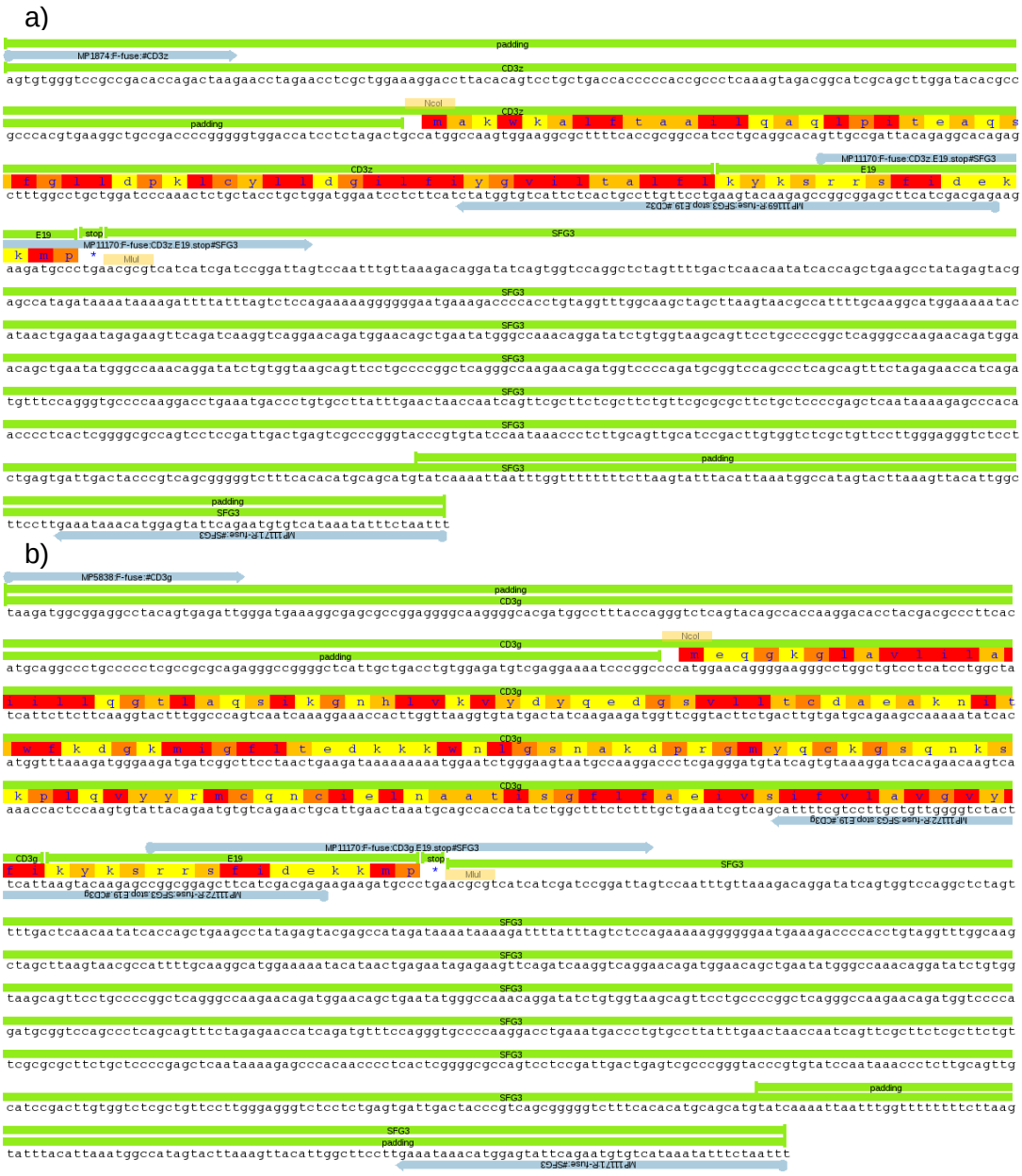


Figure 54 – Construct design for CD3 components cloned with E19 retention sequence. a) CD3ζ; b) CD3γ. Blue arrows indicate oligonucleotide primers used for fragment amplification, multi-coloured blocks denote amino acid sequence.

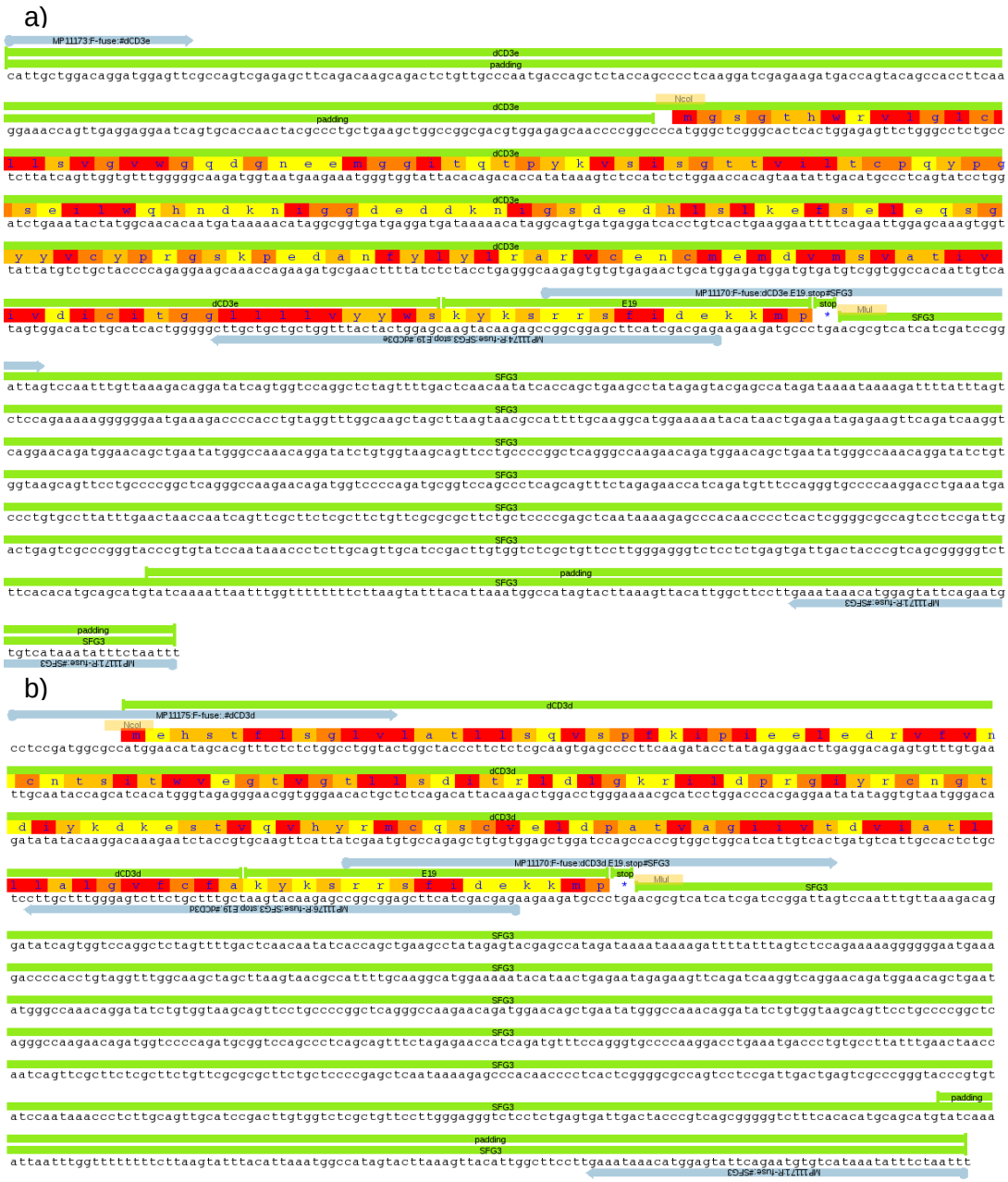
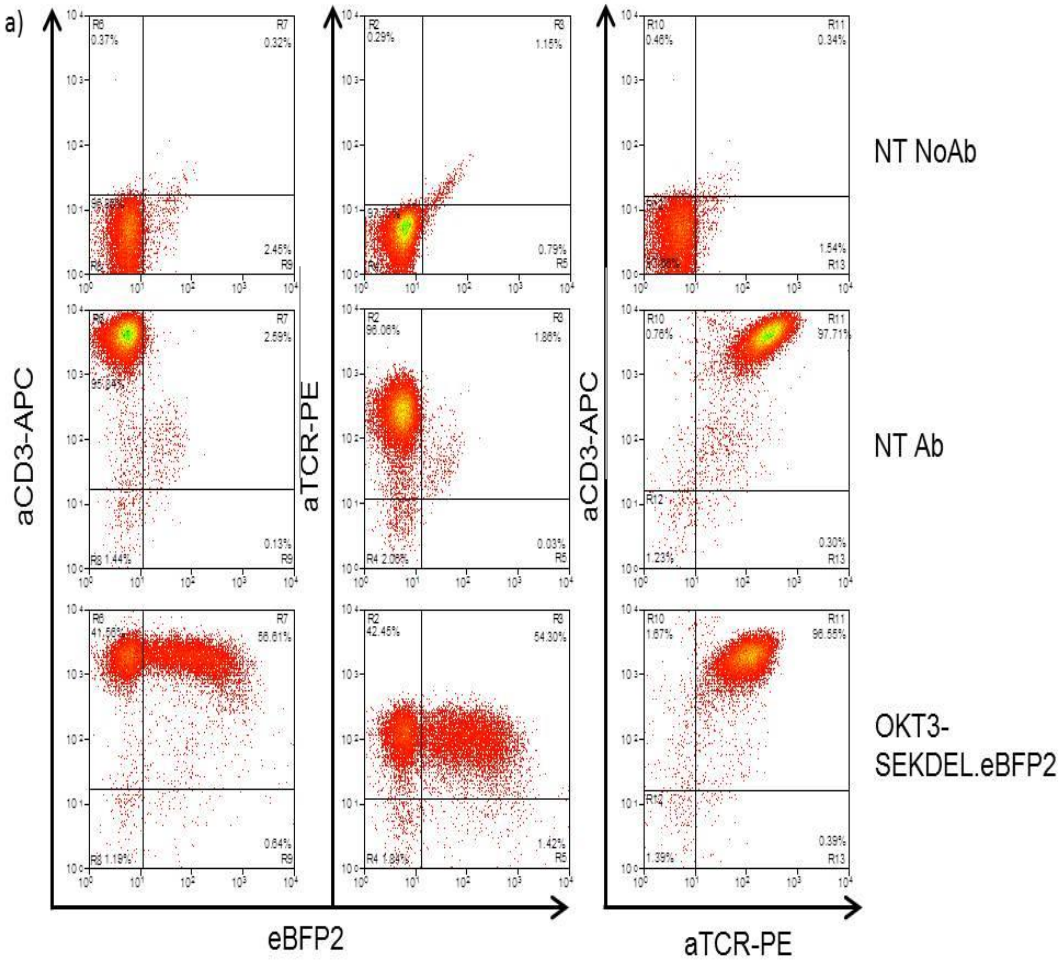
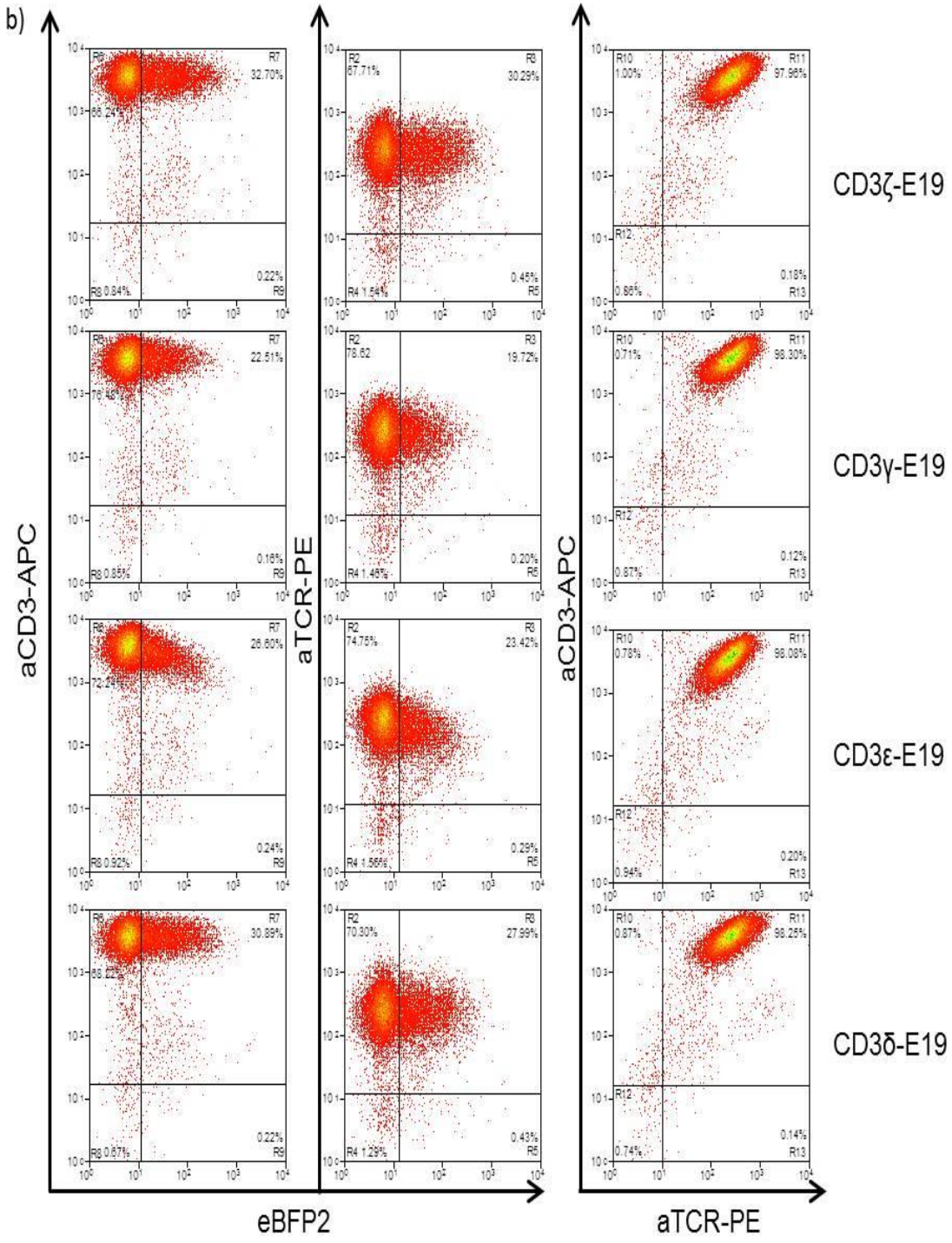


Figure 55 – Construct design for CD3 components cloned with E19 retention sequence. a) CD3ε; b) CD3δ. Blue arrows indicate oligonucleotide primers used for fragment amplification, multi-coloured blocks denote amino acid sequence.

Figure 56 – Designs for CD3 constructs with the 'KKAA' retention sequence attached. From top to bottom, CD3ζ, CD3γ, CD3ε, CD3δ. Blue arrows indicate oligonucleotide primers used to amplify CD3 components from template DNA. Multi-coloured blocks denote amino acid sequence.

These retention sequences were assembled with each of the individual CD3 subunits as shown, before retroviral supernatant was generated, which was the used to transduce donor PBMCs. The OKT3-SEKDEL construct was used as a control for comparison, and as before, a slight drop in TCR expression was seen, along with a slight drop in CD3 expression (**Figure 57**).




b)
aCD3-APC
eBFP2
aTCR-PE
aCD3-APC
aTCR-PE
CD3ζ-E19
CD3γ-E19
CD3ε-E19
CD3δ-E19

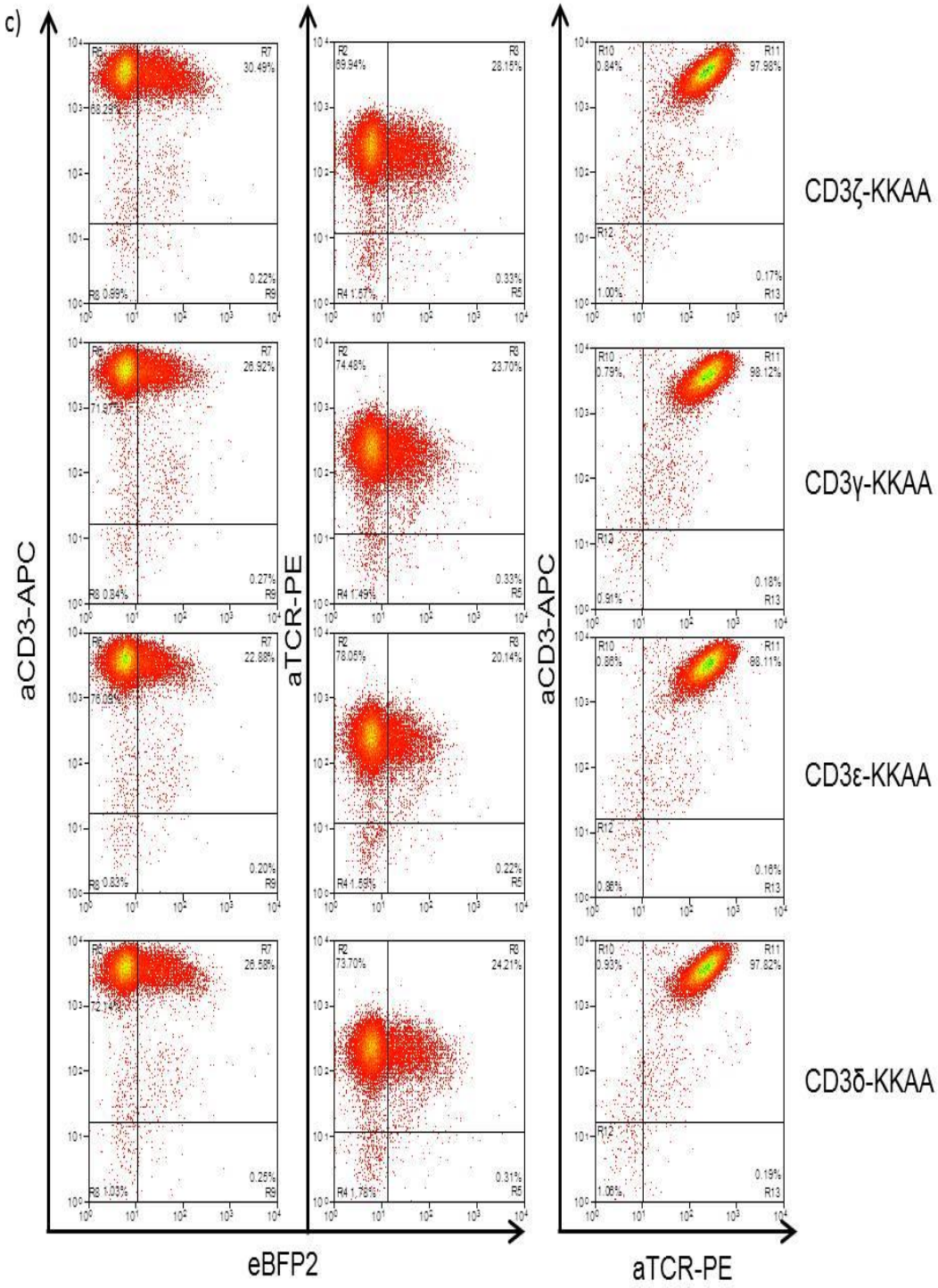


Figure 57 – Testing CD3-ER retention constructs in donor PBMCs. a) controls. b) CD3 constructs with E19 retention sequences. c) CD3 constructs with KKAA retention sequences. Cells were stained with aCD3-APC and aTCR-PE with eBFP2 marker gene. NT – non-transduced, Ab – antibody.

This data is summarised in **Figure 58**, which shows the MFI of the cells that expressed high levels of eBFP2. From this, there looks to have been a small

level of retention caused by the CD3ε subunit attached to the E19 retention sequence, but this was minimal and it was concluded that it was not sufficient enough to take this strategy further. Again, as with the TCR ER retention, it is probably due to levels of CD3 subunit expression far exceeding that of the transgene expression. What is supporting this conclusion is that both CD3 and TCR expression follow each other. Two alternative explanations may be that the CD3 constructs were unable to be incorporated into the TCR-CD3 complex before assembly, or that they did incorporate into the TCR-CD3 complex, but did not affected cell surface expression.

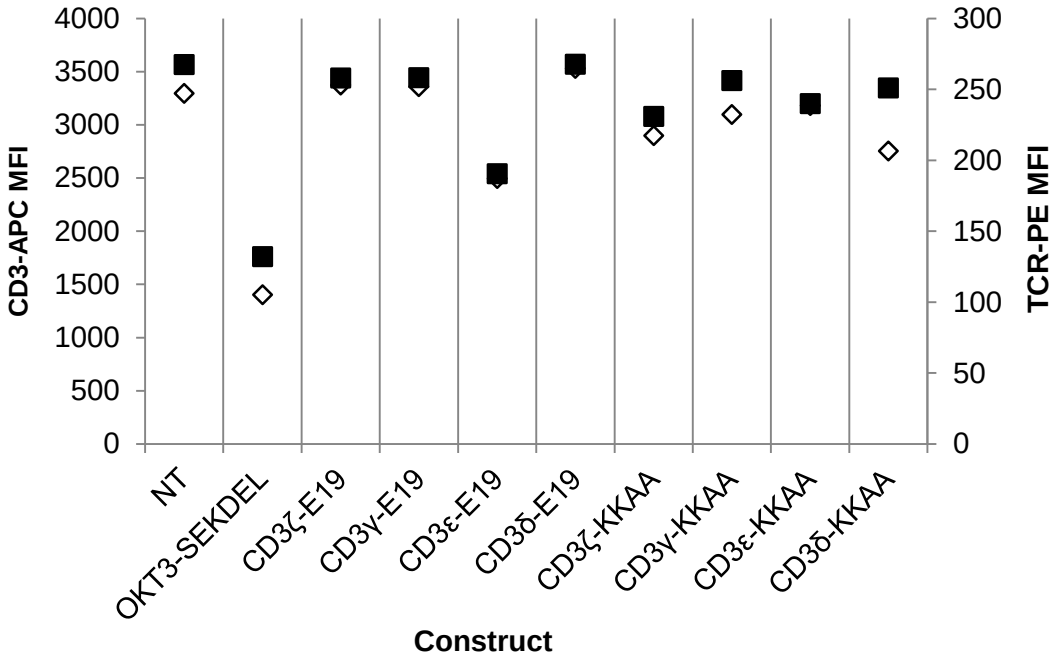


Figure 58 – MFI of high-expressing eBFP2 cells transduced with various constructs. Left hand x-axis – CD3-APC MFI (black squares); right-hand axis – TCR-PE MFI (black open diamonds). NT – non-transduced. Repeated once.

5.4.5 Inhibition of TCR expression by expression of TCR constant chains

Since the retention strategies did not have much success, it was decided to try another approach by disrupting the TCR assembly. There was a possibility that if expression of the TCR constant chain ectodomains was achieved, then these might pair with the relevant TCR alpha or beta chain, preventing correct

pairing with the TCR alpha and beta chains and subsequent cell surface expression. Although not complete, some retention had previously been seen with the ‘SEKDEL’ sequence, so constructs were made containing the TCR alpha (TRAC) and TCR beta (TRBC) constant regions alone and constructs were designed that contained the constant region and the ‘SEKDEL’ sequence (**Figure 59**-**Figure 61**).

a)

b)

Figure 59 – Designs for TRAC constructs. a) TRAC construct on its own; b) TRAC construct linked to the ‘SEKDEL’ ER retention sequence. Blue arrows indicate oligonucleotide primers used to amplify DNA fragments, multi-coloured blocks denote amino acid sequence.

a)

b)

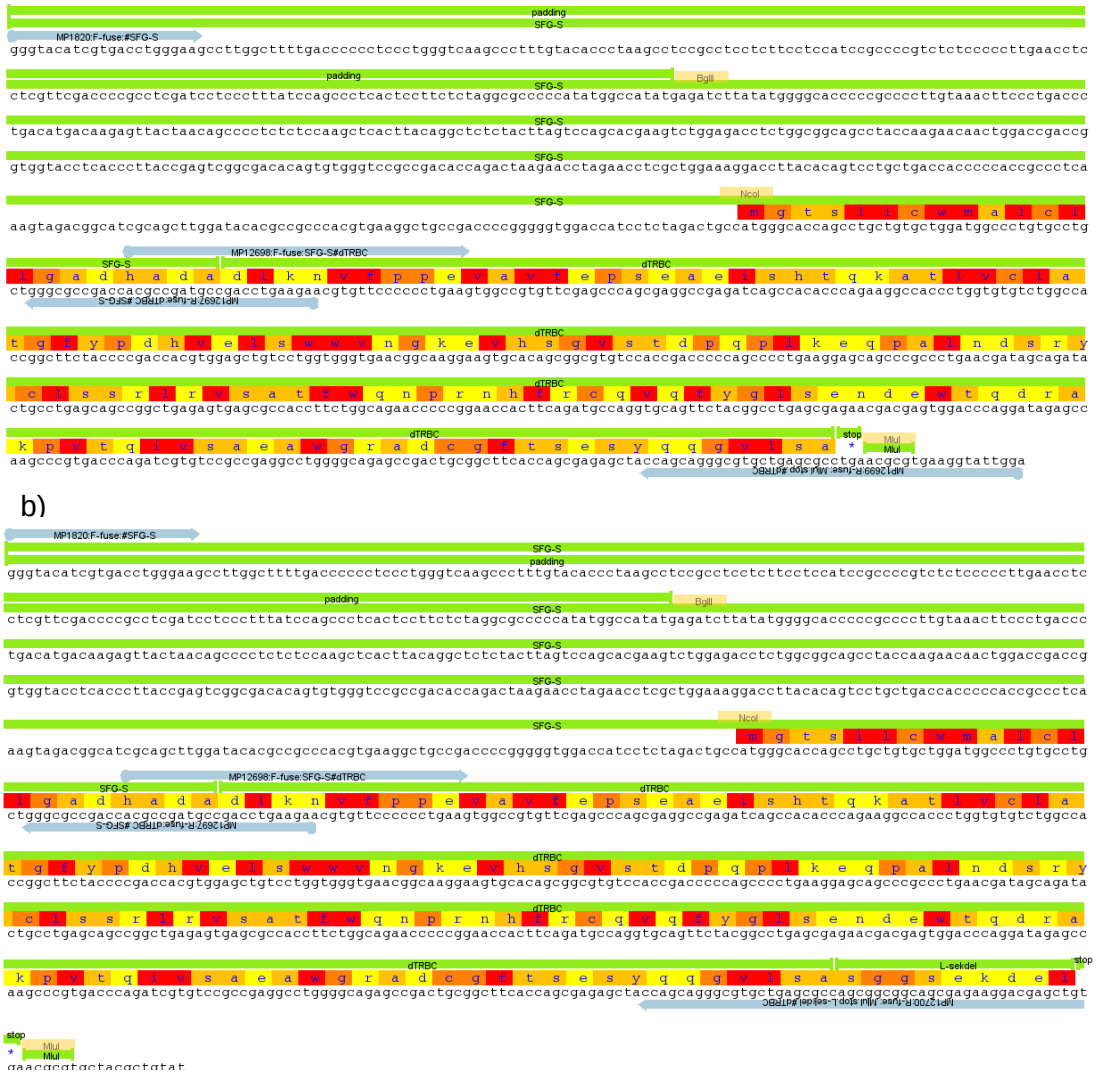


Figure 60 – Designs for TRAC constructs. a) TRBC construct on its own, b) TRBC construct linked to the ‘SEKDEL’ ER retention sequence. Blue arrows indicate oligonucleotide primers used to amplify DNA fragments, multi-coloured blocks denote amino acid sequence.

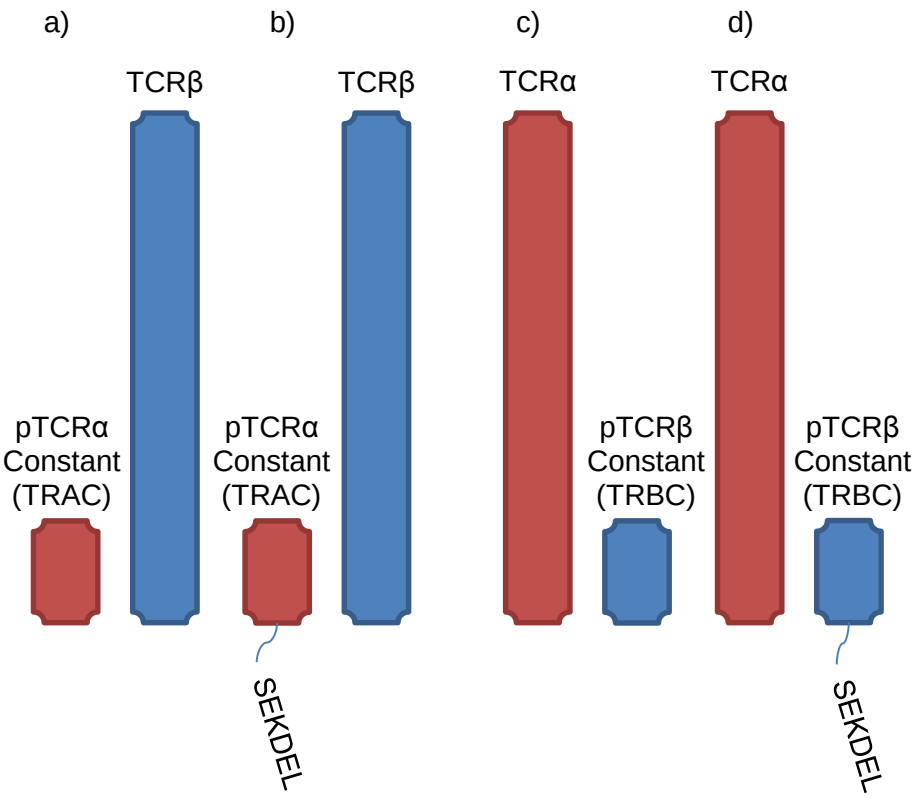


Figure 61 – Depiction of how the constant chains of the TCR might bind to the corresponding TCR chain and retain them in the ER. a) TCRα constant region binding to TCRβ chain. b) as a), but with 'SEKDEL' sequence attached. c) TCRβ constant chain binding to TCRα chain. d) as c), but with 'SEKDEL' sequence attached.

It has also been suggested that a splice variant of the PTCRA may, as a heterodimer, be degraded by the ER. This splice variant is expressed without the extracellular Ig-like domain (von Boehmer 2005). If this pairs with the TCRβ chain, then this splice variant might promote TCR degradation as a result if expressed more highly. Therefore, another strategy was to express a splice variant of the invariable chain on its own (**Figure 62**). These constructs were all assembled and transduced into Jurkat cells to determine efficacy (**Figure 63**).

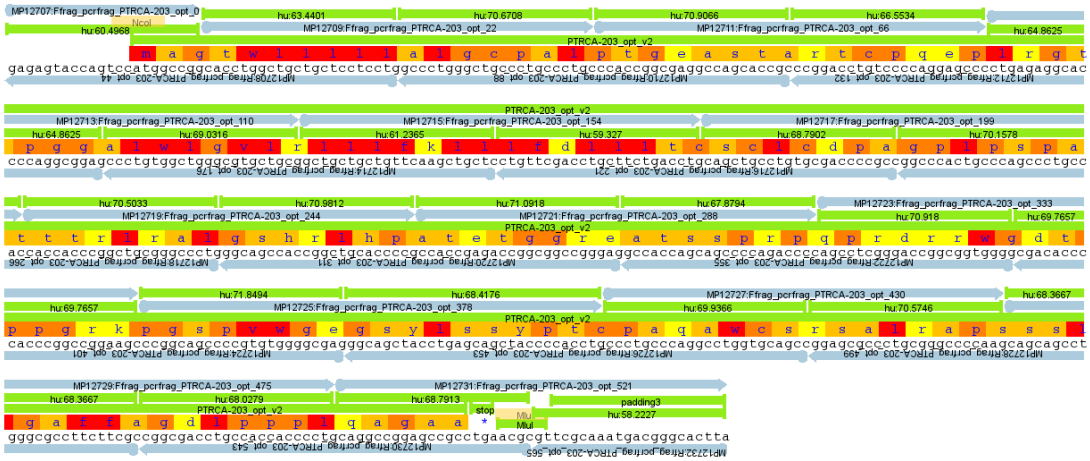


Figure 62 – Gene assembly design for PTCRA-203 splice variant. Blue arrows indicate oligonucleotides used for assembly; multi-coloured blocks denote amino acid sequence.

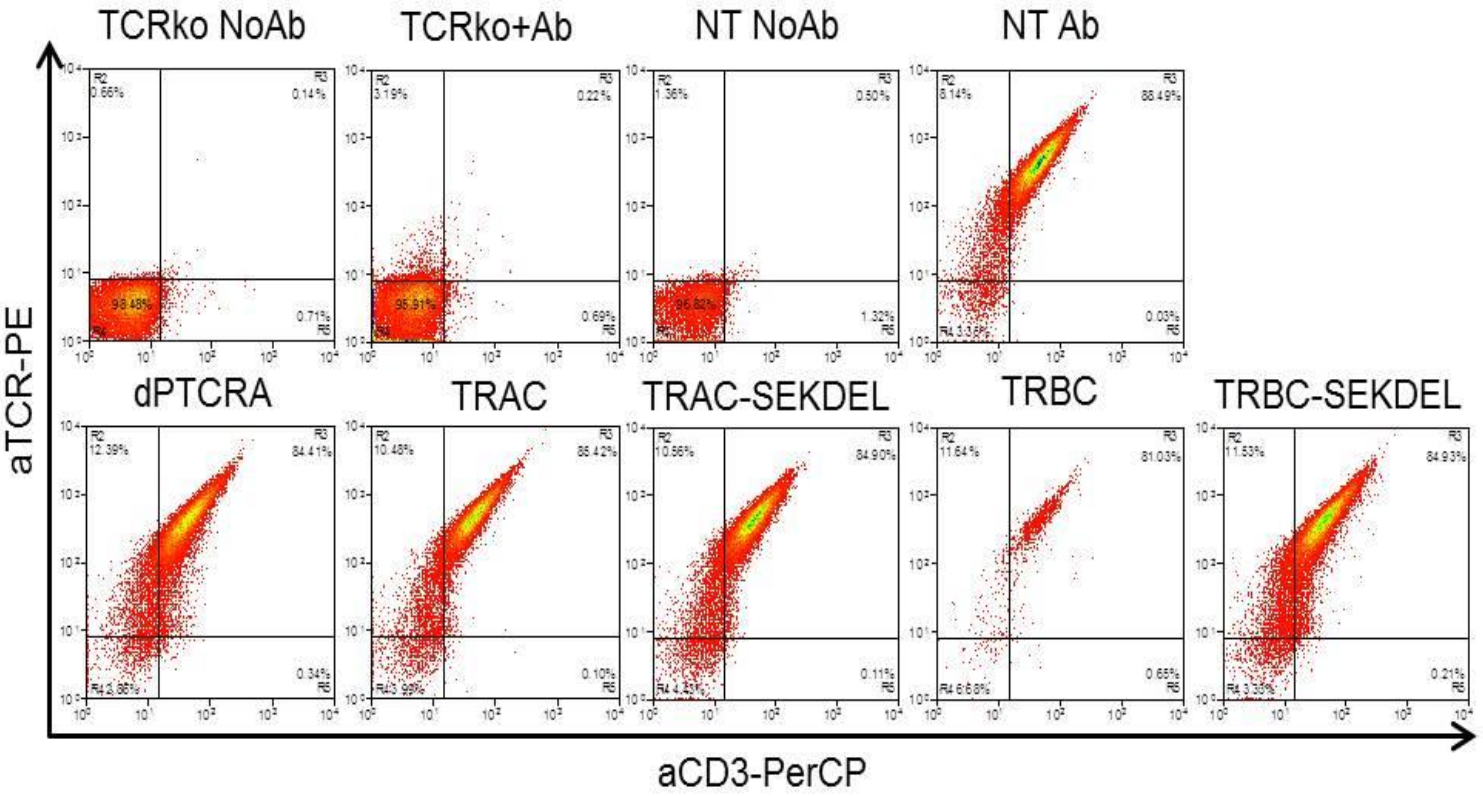


Figure 63 – Testing TCR constant region constructs in Jurkat cells. Top row – TCR knockdown Jurkat cells; NT – wild-type, non-transduced Jurkat cells. Bottom row shows wild-type Jurkat cells transduced with the relevant constructs. Truncated PTCRA construct was included in an attempt to interfere with TCR assembly. Cells were stained with aTCR-PE and aCD3-PerCP antibodies. Repeated once.

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As with previous strategies, no clear effect was seen with these constructs. Again, the likelihood is that the levels of TCR expression are far exceeding those of the transgenes tested so far. The PTCRA molecule is involved in thymic development and therefore may not have a role in mature T lymphocytes. Regarding the constant chain constructs, these may not have worked either due to not having high enough expression to match TCR expression, or the proteins may not have been able to interact with the TCR before it has already assembled and folded with the relevant chain.

5.4.6 Knockdown of the TCR by expression of a bacterial superantigen

A different strategy was to use a bacterial superantigen to knockdown the TCR. Bacterial superantigens are protein toxins that cross-link to the TCR and HLA class II in order to cause T cell stimulation, resulting in toxic shock syndrome (TSS) (Proft et al. 2000). There are numerous superantigens that have been described, most of which are derived from either *Staphylococcus aureus* or *Streptococcus pyogenes*. SMEZ-2 is found in *S.pyogenes* and has one of the highest potencies characterised. (Fraser & Proft 2008). Since superantigens bind to both the TCR and to HLA Class II, it was thought that using this molecule with a retention signal may lead to knockdown of the TCR. Therefore, SMEZ-2 was assembled with the 'SEKDEL' ER retention sequence and cloned into a SFG vector containing the eGFP marker gene (**Figure 64**). These constructs were transduced into donor PBMCs along with a control HLA-A2 construct, also with the eGFP marker gene (**Figure 65**). There are possible limitations to this strategy, including immunogenicity and inducing TSS upon T cell death, which would have to be taken into consideration.

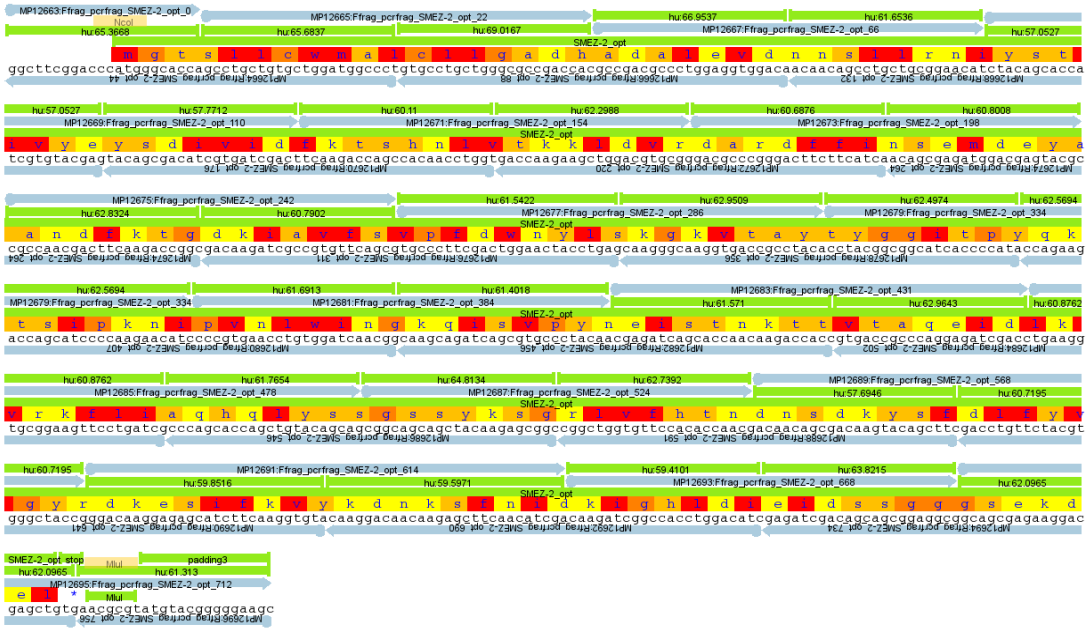

Figure 64 – Gene assembly design of SMEZ-2 construct cloned with ‘SEKDEL’ ER retention sequence. Blue arrows indicate oligonucleotides used in gene assembly; multi-coloured blocks denote amino acid sequence.

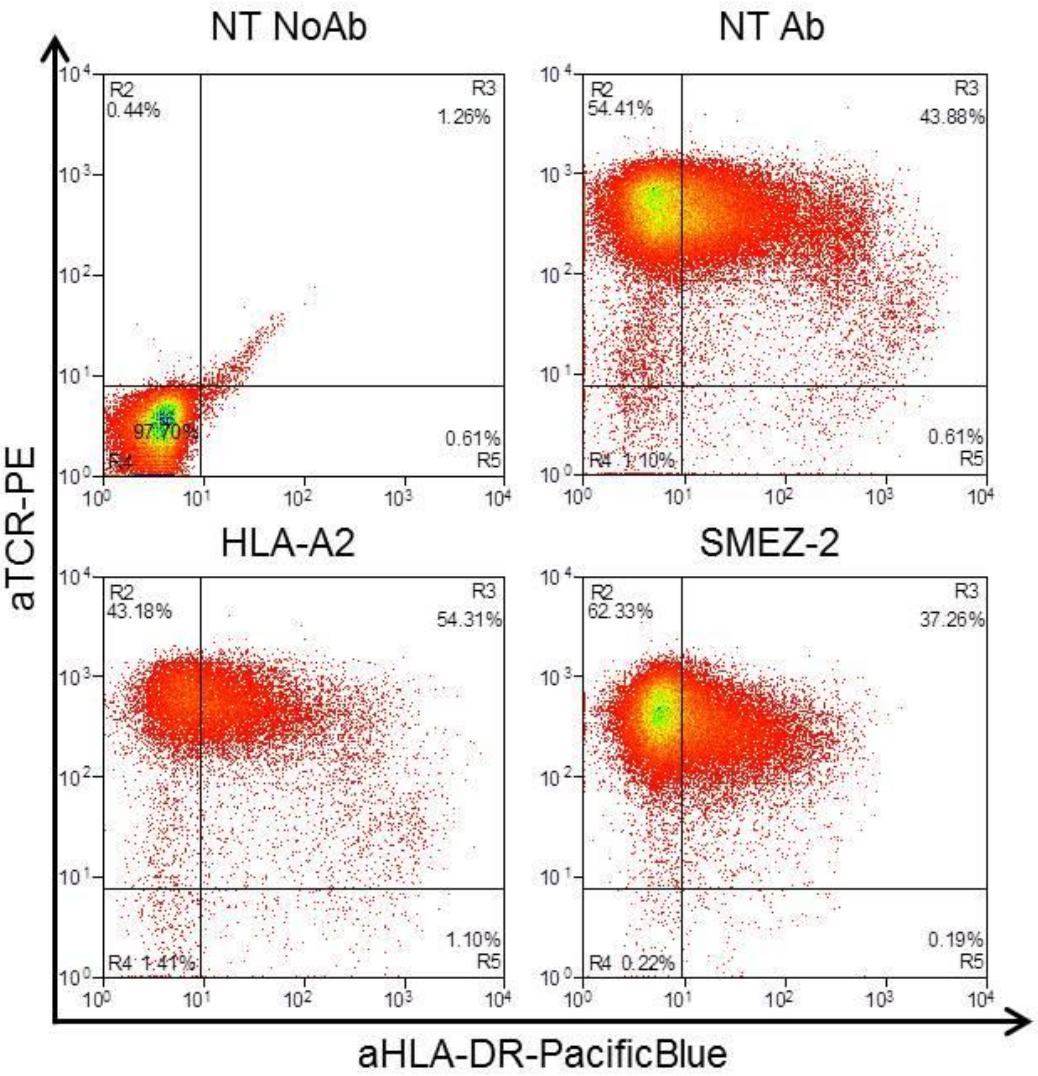

Figure 65 – Testing knockdown of TCR and HLA class II knockdown in donor PBMCs using a bacterial superantigen, SMEZ-2. Cells were stained with aTCR-PE and aHLA-DR-Pacific-Blue antibodies. NT – non-transduced; Ab - antibody. Constructs used were HLA-A2.I2.eGFP and SMEZ_2-SEKDEL.I2.eGFP. No difference seen between GFP positive and negative cells for the SMEZ-2 construct. Repeated once.

As can be seen, there is little to no effect on TCR expression and HLA Class II expression (represented by aHLA-DR antibody staining). There appears to be a slight shift downwards of TCR expression, but this is negligible. Even if this strategy had been successful, there would have been the obstacle of avoiding the possibility of TSS when using this in *in vivo* model experiments. As this strategy failed, it was decided to try another strategy.

5.5 Inhibition of TCR signalling by a null CD3

As targeting the TCR directly did not prove to be effective, it was decided to target TCR signalling. CD3 had been previously modified by attaching ER retention sequences to the various components; this strategy was aimed at preventing the initiation of the signalling cascade triggered upon TCR-CD3 activation. The CD3 components contain an extracellular domain linked by a transmembrane domain to an ITAM. These components associate with the TCR through specific amino acid residue interactions present in the transmembrane domain (**Figure 66**). Therefore, the plan was to introduce a transgene that contained both the extracellular and transmembrane domains, thereby allowing for TCR-CD3 assembly, but without the ITAMs attached, meaning that if the transgenic CD3 components associated with the TCR, upon activation, there would be no initiation of the signalling cascade. The construct was designed to contain all four components of the CD3 molecule (δ, ε, γ, ζ) separated by the FMDV 2A self-cleaving peptide, with a truncated CD34 molecule used as the marker gene (**Figure 67**).

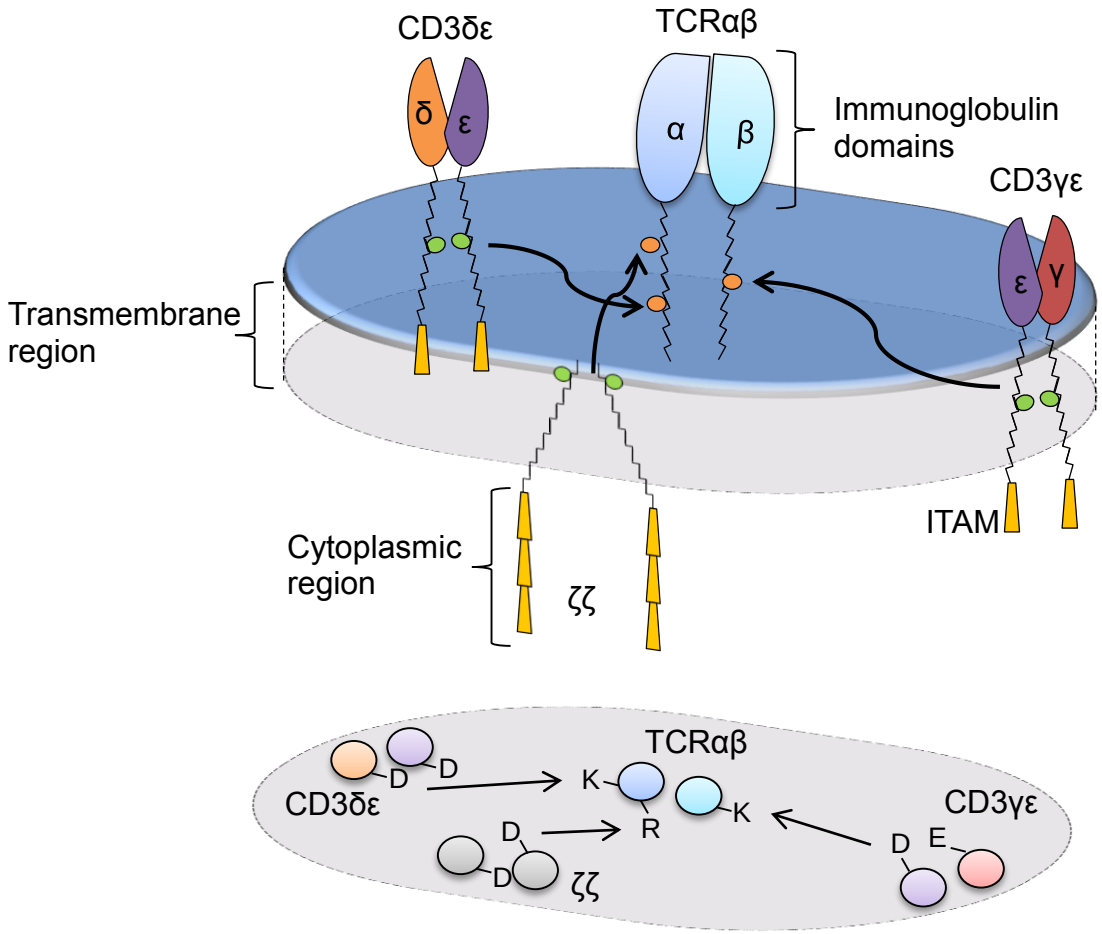

Figure 66 – Association of dimeric CD3 components with the TCR. Top diagram shows residue-specific interactions between transmembrane regions of the various components. Orange circles show basic residues; green show acidic residues. Bottom diagram shows 2D representation of top diagram with residue annotation. Adapted from Call & Wucherpfennig (2007).

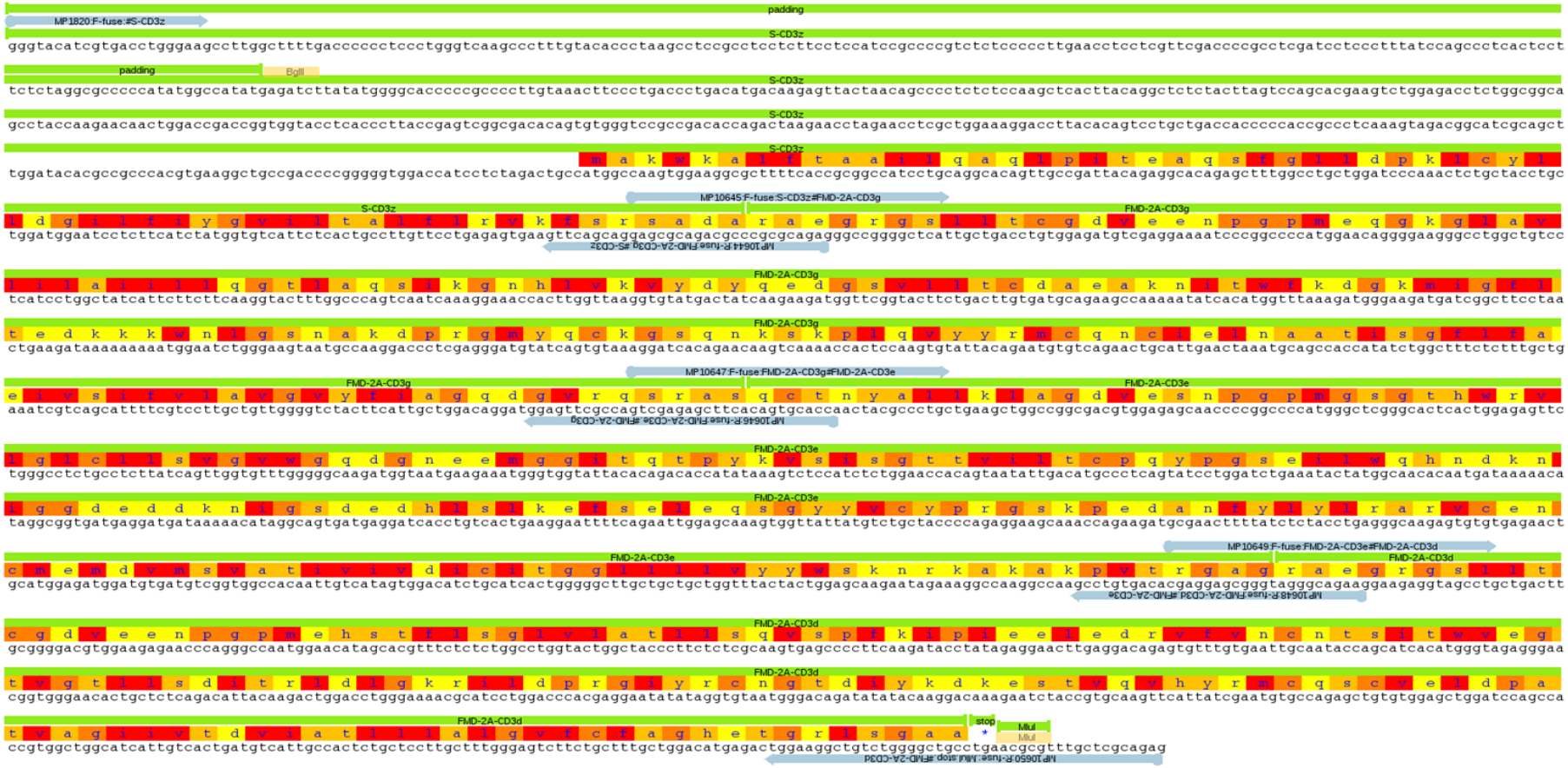

Figure 67 – Design of ‘null’ CD3 construct containing all four CD3 components, without ITAMs, separated by the FMDV 2A self-cleaving peptide. This transgene was ligated into a SFG vector containing the truncated CD34 as the marker gene using the restriction sites shown.

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This construct was successfully assembled and correct sequencing was verified, but was not able to be shown to be functional within the time frame possible. This would be revisited in the future.

5.6 Knockdown of the TCR by gene-editing TALENs

5.6.1 Ways of modifying the genome of a cell

Since the approach of targeting the TCR at the protein level has not been successful, it was decided to try and achieve knockdown of the TCR by targeting it at the gene level. There are several strategies that have been demonstrated to have the ability to edit the genome of a cell, including ZFNs, MNs and TALENs. These three methods of genome-editing are based on nuclease-mediated activity. Parts of the molecules direct the nucleases to the target site and the nuclease domain(s) cause a DSB in the DNA; this can be used to either remove sections of DNA or to insert a gene into the genome (Alwin et al. 2005; Rouet et al. 1994). A DSB stimulates the cellular machinery to induce homologous recombination (HR) to repair the DSB.

Zinc fingers are a family of DNA-binding molecules that bind to a specific DNA sequence around 9bp in length. These have been fused to the endonuclease domain of the *FokI* restriction enzyme, giving rise to ZFNs, which have been demonstrated to cleave DNA at a specific site both *in vitro* and *in vivo* (Bibikova et al. 2003; Urnov et al. 2005). These chimeric ZFNs have also been designed to act in pairs and will not induce a DSB without dimerising with the other ZFN of the pair present. This is achieved by using a nuclease that requires dimerization for cleavage activity to be initiated (Vanamee et al. 2001). This technology allows for specific sites to be targeted, but it does have the drawback of relying upon using zinc fingers that are already in existence in nature and have been characterised. Unlike MNs and TALENs, these molecules cannot be modified to target any sequence of DNA.

MNs are yeast-derived molecules that can induce homologous recombination (HR) at up to 1000-fold in cultured cells. They are highly-specific and provide a scaffold upon which gene-editing can be built. Based on sequence and structural motifs, MNs can be divided into five main families, LAGLIDADG, GIY-YIG, HNH, His-Cys box and PD-(D/E)XK (Orlowski et al. 2007; Zhao et al. 2007). MNs are able to target a region of DNA 14-40bp in length, and then subsequently create a DSB in that region. Since the recognition sequence is so long, the MNs can usually tolerate polymorphisms in the site with little or no loss in the cleavage activity (Arnould et al. 2011). MNs can also be modified to target different DNA sequences. Mutations of certain residues has resulted in modification of target site cleavage without loss of cleavage activity, but the amount of modification without cleavage activity loss is still limited (Seligman et al. 2002; Sussman et al. 2004; Doyon et al. 2006).

TALENs are transcription activator-like effector molecules, derived from the plant pathogen *Xanthomonas*, fused to the *FokI* endonuclease. Each TALE molecule has repeats of highly conserved regions, which are usually 33-35 amino acids long. These repeats are responsible for DNA binding; one repeat unit binds to one DNA nucleotide. The unique feature of these molecules compared to MNs and ZFNs is that they are customisable by modification of the polymorphic amino acids. Within each repeat, the two residues at positions 12 and 13 are the variable residues determining specificity, and are called RVDs. These repeat units allow for a DNA sequence of 15-30 nucleotides to be recognised as the DNA binding domain of each TALE. Two TALEN molecules are required in order for dimerization of the *FokI* nuclease in order for cleavage activity to be activated (Miller et al. 2011; Moscou & Bogdanove 2009; Boch et al. 2009; Morbitzer et al. 2010; Mussolino et al. 2011). Cellectis Bioresearch kindly allowed the use of their TRAC-targeted TALEN molecules in order to investigate this method for achieving TCR knockdown.

5.6.2 TCR knockdown by TRAC TALENs

Cellectis Bioresearch provided mRNA containing engineered TALENs directed against the TCR alpha chain. Donor PBMCs were stimulated with CD3/CD28 Dynabeads for 48hrs before the TCR alpha chain TCRko TALEN mRNA was introduced by electroporation. Cells were recovered before being stained to determine the level of TCR knockdown at 24hrs (no effect seen) and 48hrs (**Figure 68**).

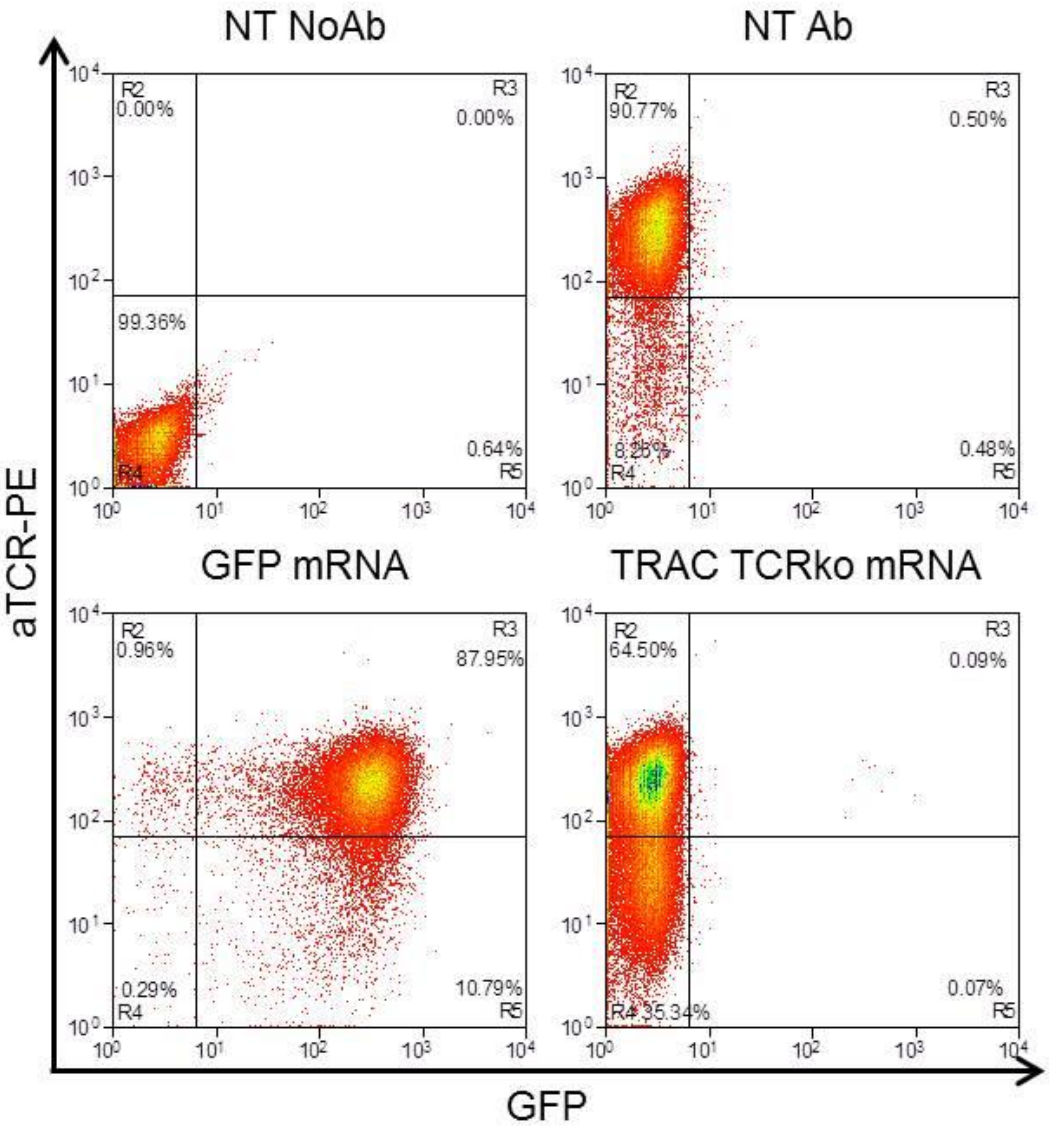

Figure 68 – Donor PBMCs electroporated with TRAC TCRko mRNA. Cells were stimulated with CD3/CD28 Dynabeads before electroporation with GFP control mRNA or TRAC TCRko TALEN mRNA, followed by recovery in cRPMI without cytokines. Cells were stained with aTCR-PE antibody 48hrs after electroporation before FACS analysis. NT – non-treated cells; Ab – antibody. Repeated once.

As can clearly be seen, some knockdown of the TCR has been achieved. In order to get a clearer picture on the level and amount of knockdown achieved, it is easier to look at the histograms derived from **Figure 68** (**Figure 69**).

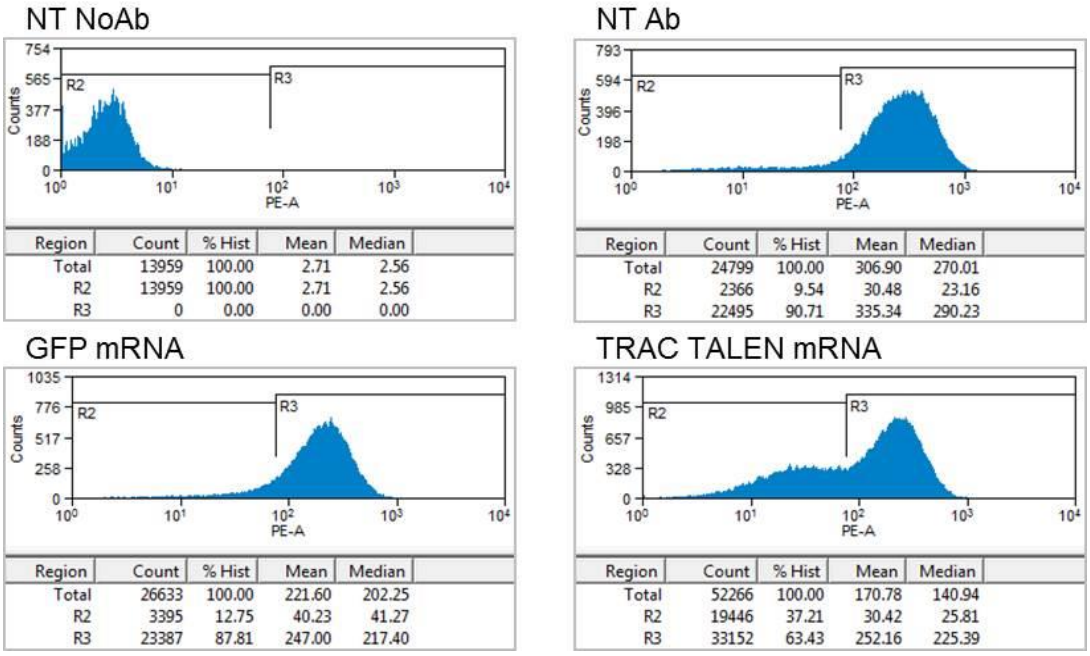

Figure 69 – Histograms with statistics taken from **Figure 68**, showing level of TCR knockdown as a result of treatment with TRAC TALEN mRNA. NT – non-transduced; Ab – antibody. Repeated once.

As can be seen, the proportion of cells in R10 increases from 14.39% in the aTCR-PE stained NT PBMCs, to 39.00% in the TRAC TALEN electroporated PBMCs, an increase of around 25%. The overall MFI also drops from 294.01 to 169.10. This shows that it is possible to reduce TCR expression on T cells, although not fully.

5.6.3 Introduction of a CAR to TCR^{lo} cells

It was possible to cause a reduction in TCR expression following treatment with TRAC-targeted TALENs. If these T cells were left in culture though, the TCR^{lo} T cells would most likely die as a result of a lack of stimulation through the remaining TCRs expressed on the T cell surface. These TCR^{lo} T cells need

a signal to induce proliferation and survival. Therefore, it was decided to see if it was possible to subsequently transduce TALENised T cells with an aCD19 CAR. PBMCs were isolated from a healthy donor and stimulated with aCD3 and aCD28 Dynabeads for 48hrs before electroporation with TRAC TALEN mRNA. Immediately after electroporation, the cells were transduced with an aCD19-CAR (with RQR8 sort-suicide gene used as a marker). Cells were left to recover for 48hrs after electroporation and transduction before being analysed by FACS (**Figure 70**).

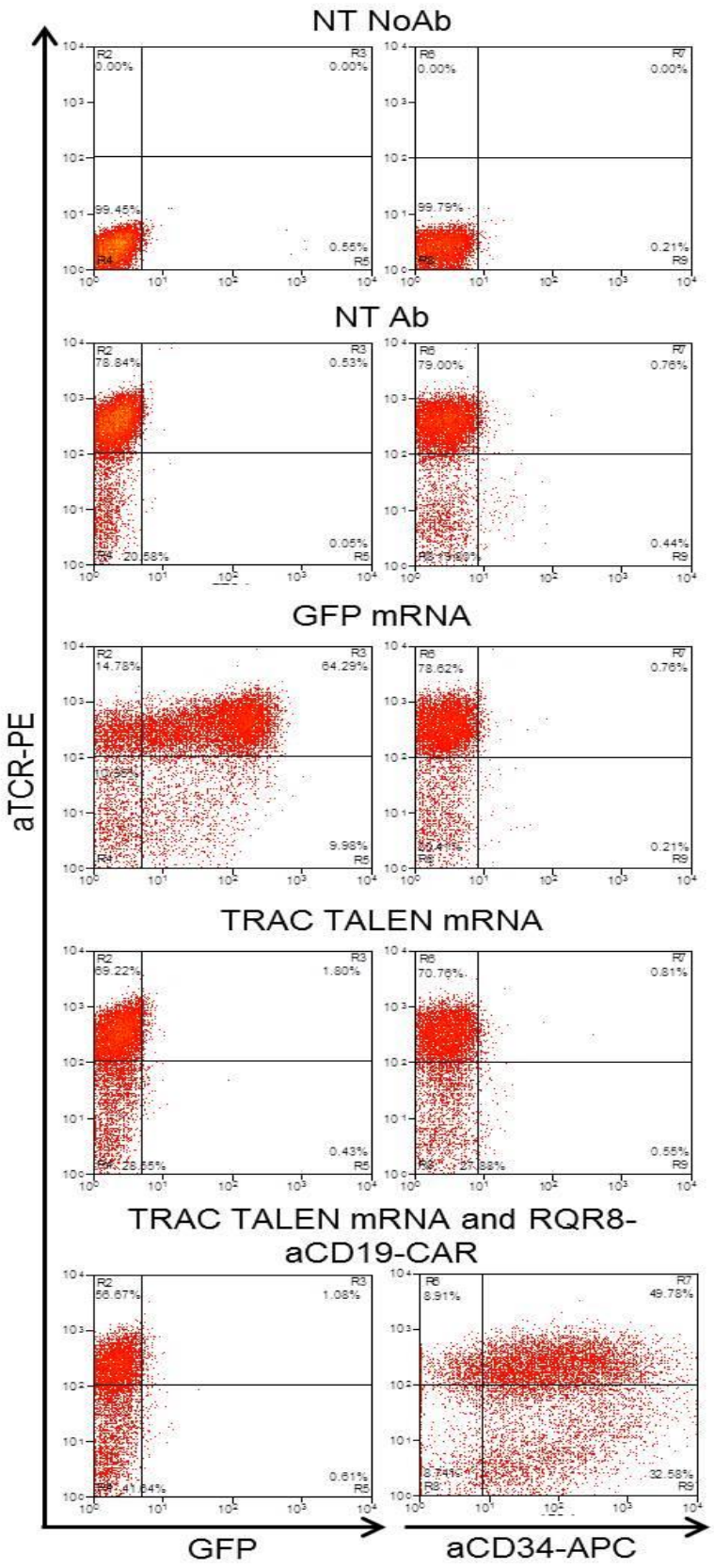

Figure 70 – Donor PBMCs electroporated with TRAC TALEN mRNA and transduced with an RQR8-aCD19-CAR. Before modification, PBMCs were stimulated with aCD3 and aCD28 Dynabeads for 48hrs. After treatment, PBMCs were recovered for 48hrs before FACS analysis using aTCR-PE and aCD34-APC antibodies. NT – non-transduced; Ab – antibody. Repeated once.

From this experiment, it is clearly possible to obtain T cells that have reduced levels of TCR expression alongside CAR expression. Over 82% of the cells that have been treated with TALEN mRNA and aCD19-CAR are CD34 positive. Of that 82%, 32.58% are in R9 (lower right quadrant). These cells make up over 15% of the total number of cells analysed in this FACS analysis sample.

5.6.4 MACS sorting TCR^{lo} T cells

Following on from this, the next logical step was to try and sort the TCR^{lo} T cells to obtain a pure homogenous population of T cells. There were two options in terms of ways to sort cells – either through using the MOFLO XDP (BeckmanCoulter), or by using MACS beads (Miltenyi). MOFLO XDP cell sorting uses FACS analysis as the basis for cell sorting, and therefore allows cells to be sorted based on various cell surface markers or fluorescent marker genes, but it is done in a non-sterile environment and therefore requires the use of antibiotics in order to try and prevent bacterial or fungal contamination during the cell sorting process.

MACS sorting can be done in a sterile environment, such as a class II biological safety cabinet, and as the aim for these cells is for them to ultimately be used in patients, this method was deemed to be the most appropriate. Miltenyi did not have aTCR MACS beads available, so a two-step method was used where aTCRαβ-biotin antibody was used to bind the T cells, followed by incubation with streptavidin MACS beads. The bead-labelled cells were then run through a MACS column, with the positive and negative fractions being separated and recovered for 48hrs before they were analysed by FACS (**Figure 71**).

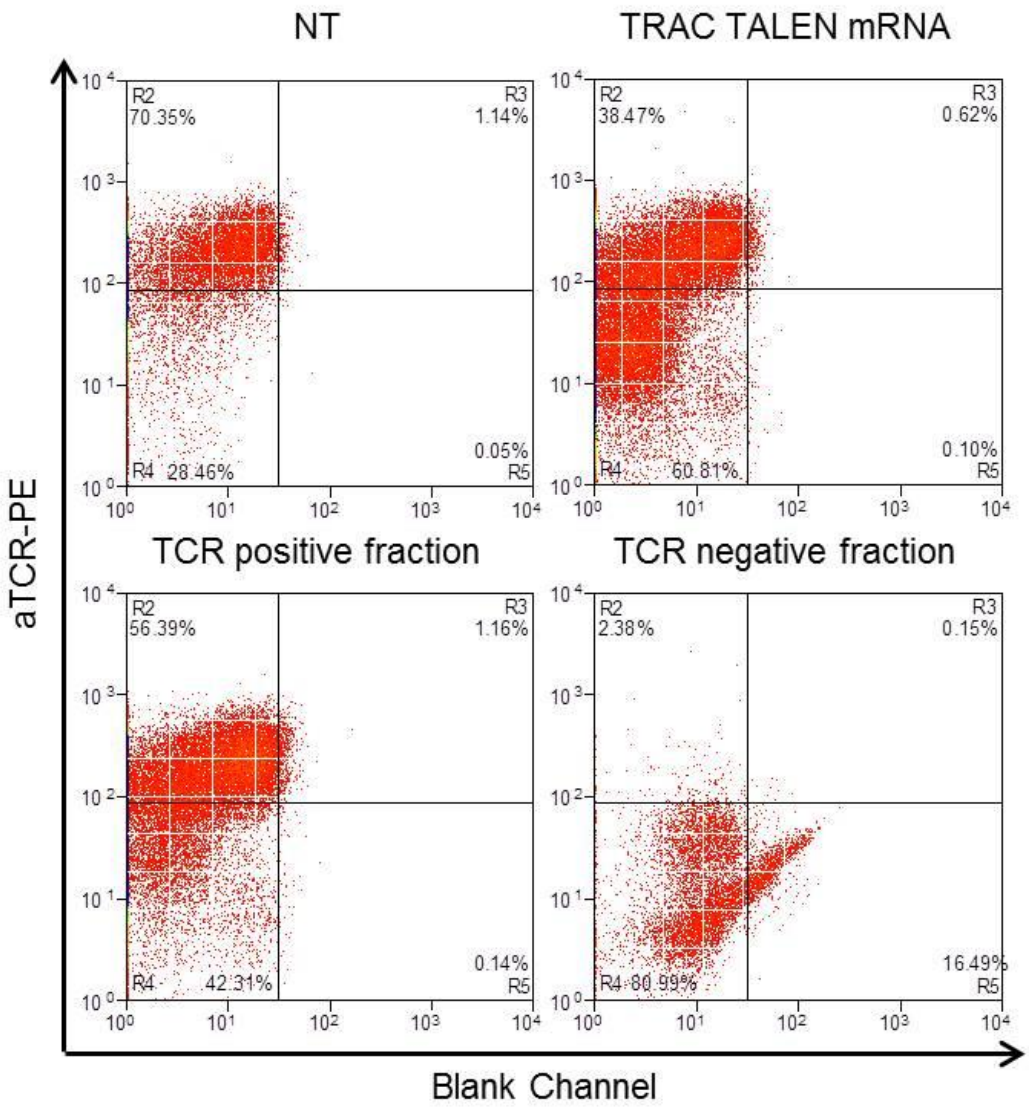

Figure 71 – MACS sorting of TALENised T cells. Cells were stained with aTCRαβ-biotin antibody before being incubated with Streptavidin MACS beads. Cells were then passed through a MACS column, with the flow-through fraction labelled as the TCR negative fraction and the bound fraction as the TCR positive fraction. NT – non-treated. Repeated once.

As can be seen, the viability of the cells is maintained in the TCR positive fraction, but the TCR negative fraction was very low, around 1% based on a FSC/SSC live/dead gate. Therefore it can be said that the MACS selection has worked, but the viability of the cells has been lost.

5.7 Conclusions

In conclusion, after trying multiple methods, partial knockdown of the TCR was achieved. Several key discoveries were made during this process, which could lead to further investigation and further attempts to modify TCR and/or T cell signalling in order to provide alternative immunotherapeutic treatments.

To begin with, aTCR antibodies were used to target the TCR. These aTCR antibodies were shown to bind to the TCR, and were then modified with an ER retention sequence attached to the C terminal end of the protein. This was aimed at causing the naïve TCR to be retained in the ER and prevent cell surface expression. What was seen was partial apparent knockdown of the TCR in both the transduced and non-transduced cells. After using techniques such as co-cultures and transwells, the most likely explanation for this phenomenon is that the aTCR-SEKDEL constructs were binding to the TCR but were not able to cause ER retention. When expressed on the cell surface, the TCR-aTCR-SEKDEL complex blocked the fluorescence-conjugated antibody from binding, therefore giving an apparent knockdown in cell surface expression of the TCR. It also appeared that the cell surface expressed aTCR-SEKDEL constructs were able to bind TCRs on the cell surface of neighbouring T cells, resulting in the apparent knockdown of TCR on non-transduced cells. The key discovery here was that this showed that the constructs had the possibility of being flexible enough to bind to TCRs in a paracrine and autocrine way, which could lead to further investigation.

As the above strategy was not highly effective, and because there were other unexpected effects seen, the CD3 molecule was targeted next, using the ‘KKAA’ and E19 adenoviral ER retention sequences attached to the various CD3 components. No effect was seen by any of these constructs except for the CD3ε constructs, which only gave a small hint of any TCR knockdown effect; less so than previously seen with the aTCR-SEKDEL constructs. As this strategy failed, a slightly different tact was taken for the next strategy,

which was to add an ER retention sequence to the constant regions of the TCRα and TCRβ chains, with the aim of disrupting correct folding and cell surface expression. Of the three main ER retention strategies tested, this was the least effective and did not show any signs of success.

Still sticking with the strategy of targeting the TCR and its signalling components directly, a construct containing 'null' CD3 components was designed and created. The CD3 components all lacked their ITAMs, with the aim of these components binding to the TCR in a competitive manner, and preventing TCR signalling and activation of the T cell. Intracellular IFNγ was used as the readout for this strategy, but unfortunately no effect was seen as a result.

The penultimate strategy attempted was to use something found in nature, a bacterial superantigen. This was cloned based on a sequence obtained from a previous study, and an ER retention sequence was attached to see if this could improve the efficacy of the construct further. Unfortunately, no effect on TCR surface expression was seen as a result of transduction with the bacterial superantigen construct, so this strategy was not pursued.

Finally, the last strategy tried was gene-editing TALEN molecules. Fortunately, these were donated from Cellectis Bioresearch, and therefore had been previously shown to cause TCR knockdown from the cell surface. The challenge was to be able to replicate this for this study, and also to build on it by adding other aspects of the project in order to combine as many aspects as possible. The replication itself was not easy, as the process of electroporation is quite a crude one, and often results in high amounts of cell death, and requires protocol optimisation in order to be able to recover as many cells as possible for downstream use. Once TCR knockdown had been shown, the TCR^{lo} cells were also transduced with an aCD19-CAR that had the RQR8 sort-suicide gene as a marker gene. Around 25% of the cells had reduced levels of TCR cell surface expression, with the MFI being reduced from 294 to 169 (a drop of nearly 50%). The aim was to try and optimise this process in order to obtain as many TCR^{lo} CAR^{hi} cells as possible, but unfortunately, it was not possible to do so. It was also shown to be possible to

sort the TCR^{lo} and TCR^{hi} populations by using MACS technology, but again, the TCR^{lo} cells had a very low viability and succumbed to cell death within 48hrs. This does show some promise, however, and since performing these experiments, it has been shown that it is possible to use TALENs to knockdown expression of the endogenous TCR in Jurkat and primary T cells at levels of 40-50% (Berdien et al. 2014).

Despite these setbacks, it has clearly been shown that it is possible to cause partial knockdown of the TCR from the cell surface, and that it is possible to transduce these cells with a CAR in order to re-direct the targeting moiety of the T cells. Work done by Torikai et al. (2012) has reversed the order of T cell modifications to introduce the CAR first, followed by the TCR knockdown strategy (in this case, ZFNs). This may be another way of overcoming issues associated with cell viability *in vitro*. This provides a firm foundation for further investigation into the development of a universal T cell that can be used in adoptive immunotherapy.

5.8 Final Conclusions

- The OKT3 and BMA031 antibodies are both able to bind to the TCR.
- The ER retention sequences, ‘SEKDEL’ and ‘KKAA’ and the E19 adenoviral retention sequence were ineffective in achieving TCR knockdown when attached to aTCR antibodies, CD3 components or the TCRα/β constant chains.
- The aTCR-SEKDEL constructs caused an unusual effect of blocking TCRs on neighbouring non-transduced cells.
- Removing the ITAMs from the various CD3 components was ineffective at causing TCR knockdown.
- The bacterial superantigen SMEZ-2 was unable to cause TCR knockdown.
- TALEN molecules directed towards the TCRα constant chain were effective at causing partial TCR knockdown in around 25% of treated cells.

- TALENised T cells could be transduced with a CAR.
- TCR^{lo} and TCR^{hi} fractions were able to be separated by the use of MACS technology.

Chapter Six: Discussion

6.1 Study conclusions

With the efficacy ‘ceiling’ drawing closer for traditional chemotherapeutic agents, there has been an increased use of adoptive immunotherapy to treat cancer in the past few years. It is becoming increasingly clear that the human body can be more effectively treated by modification of cells that are already present rather than by introducing external, foreign agents. What is also becoming clear is that there are two possible routes for the potential development of adoptive immunotherapy treatments. One is the highly personalised route, whereby treatments are designed and created based on the individual’s need, which could potentially result in more effective treatment of disease. The second route is the universal route, whereby one treatment is developed in order to treat a disease in multiple patients rather than on a case-by-case basis. This is much quicker and cheaper, but has an increased risk of adverse events and unwanted side effects. That is not to say, though, that these two development routes are mutually exclusive. Ultimately, universal treatments will be modified, in terms of factors including dosage and time, in order that they may be as personal as possible. That is why many of the major pharmaceutical companies are currently investing in biological research programs as this is the new frontier where diseases, such as cancer, will be tackled in the coming years.

Adoptive immunotherapy has already been shown to be effective in numerous settings, including GvL (Bonini et al. 1997; Casucci, Perna, et al. 2013), CMV (Riddell et al. 1992, Walter et al. 1995, Einsele et al. 2002, Peggs et al. 2003), to reconstitute EBV immunity in post-transplant lymphoproliferative disease (PTLD) (Heslop et al. 1994a, Rooney et al. 1995) and in the use of TILs for metastatic melanoma (Rosenberg et al. 2008). Clearly, there is rapid progress in the development of these therapies, but they do have their limitations. The current issue with these forms of adoptive immunotherapy is that they are mainly based on personalised therapies, where the patients’ own cells have been extracted and modified before reinfusion. As a result, the cells themselves may be unhealthy due to the ongoing treatment regimen and disease load on the patient, and the time and cost of extracting, modifying,

expanding and reinfusion of patient cells is significant. As such, development of a universal T cell could circumvent many of these issues and also significantly reduce the time taken from diagnosis to initiation of adoptive immunotherapy treatment. Once a diagnosis has been made, pre-engineered T cells could be thawed, expanded *in vitro* and then delivered to a patient within a couple of weeks. As a result, this study was initiated in order to investigate the genetic engineering and manipulation of cells required to develop a universal T cell. Various avenues of molecule knockdown have been tested in primary and secondary cell lines, with the results showing that it is possible to achieve surface expression knockdown of both HLA Class I and also the TCR, as well as increasing HLA-G and RQR8 expression.

For the HLA Class I knockdown, 16 different constructs were cloned. The aim of achieving HLA Class I knockdown was to prevent the allogeneic responses triggered upon recognition of foreign HLA by the recipient. These constructs comprised various methods of achieving HLA Class I knockdown, including variants of a mutant β2m molecule, designed to interfere with the correct folding of naïve HLA Class I. A previous study has shown that, at high (μM) concentrations, a mutant β2m molecule was able to block HLA Class I molecule folding. Also demonstrated was the ability of the dominant negative β2m molecule to block T cell functional responses to peptide-loaded HLA Class I molecules (Hill et al. 2003). siRNA was also tested, two of which were replicated from previous studies (Gonzalez et al. 2005; Haga et al. 2006) and the rest which were designed using an online tool. Again, these were directed at β2m, with the aim of preventing HLA Class I assembly due to a lack of β2m in the cell. Antibodies directed to β2m were also cloned and attached to an ER retention sequence in order to prevent cell surface expression of HLA Class I. Two antibodies were replicated from a previous study (Mhashilkar et al. 2002) and one was extracted from the BBM1 hybridoma (LGC 2012). Finally, two viral proteins, US11 and ICP47 were cloned into the SFG backbone, with ICP47 previously shown to interfere with peptide loading and US11 shown to cause degradation by the proteasome as a result of ubiquitination of the HLA Class I molecule (Radosevich et al. 2003). All 16 constructs, with the exception of the viral proteins, were targeted at the β2m molecule, which is seen as

essential to the expression of HLA Class I molecule, and key to allowing exit of the HLA Class I molecule from the ER and Golgi apparatus. As with the TCR, there may have been too much β2m expressed in a cell for the constructs to be successful at achieving HLA Class I knockdown. All constructs were tested in SupT1s, with the constructs giving the most knockdown being taken forward and tested in primary cells. The two viral proteins gave the highest levels of HLA Class I knockdown in primary cells, and US11 in particular seemed to be the more effective of the two constructs.

These were tested in a functional proliferation assay in an allogeneic context, and showed evidence of a reduction in effector cell proliferation when compared to target cells that had not been modified with the viral proteins. Ideally, the results seen in the studies by Berger et al. (2000) and de la Garza-Rodea et al. (2011) would have been replicated to fully demonstrate the reduction in $CD8^+$ T cell response to HLA Class I negative cells as a result of the use of viral proteins, and this is something that would need to be worked on further. The populations of target cells used in this functional assay were homogenous as the HLA Class I knockdown strategies had been combined with the RQR8 sort-suicide gene, which allowed for sorting of HLA Class I negative cells using the Miltenyi MACS system. The HLA Class I knockdown aspect of the study was particularly pivotal as it was designed to prevent the therapeutic cells from being rejected by the recipient's immune system (Li & Sykes 2012; Game & Lechler 2002). It is clear therefore, that HLA Class I knockdown is needed in order to progress with the development of a universal T cell. It is unclear how much effect HLA Class II molecules have upon effector cell proliferation, but it is likely that HLA Class I is responsible for the majority of the allorecognition by recipient cells, as it is shown to mainly interact with the CD8 molecule on T cells (Barclay et al. 1997; Britten et al. 2002). Inhibition of this CD8-HLA Class I interaction would therefore cause the greatest amount of reduction in allorecognition. Despite this, it is highly likely that the CD4-HLA Class II interaction is also responsible for a proportion of the allorecognition. Molecules such as Class II-associated invariant chain peptide (CLIP) could be targeted, or the use of blocking antibodies could be investigated in order to interfere with HLA Class II expression and function.

To address the issue of NK-cell mediated killing of HLA Class I null cells, HLA-G has also been introduced to primary and secondary cell lines, including Jurkats, SupT1 cells and PBMCs. The isoform chosen was HLA-G1, which contained the three membrane-bound α subunits, which would associate with the β2m subunit before progressing to cell-surface expression. HLA-G1 was cloned from genomic DNA and inserted into an SFG vector containing a marker gene. Generation of retroviral supernatant and subsequent transduction into cell lines resulted in HLA-G expression that was detectable by FACS analysis. Before attempting to determine the functional effect of this strategy, it was decided to combine the HLA-G expression with the HLA Class I knockdown strategy. One possible conflict between the two was that, if HLA-G was a similarly structured molecule to HLA Class I, then the HLA Class I knockdown strategies may have impacted on HLA-G expression. From multiple tests in SupT1s and PBMCs, it appears that it is possible to co-express these two strategies, but that it did appear that the HLA Class I knockdown did cause a reduction in HLA-G expression. This may have been due to the mechanism of ICP47 blocking peptide entry via the TAP molecule. If this is the case, then there may be a suggestion that HLA-G has some previously unknown antigen presenting properties, which would warrant further investigation outside of this project. With US11, this may have caused a reduction in HLA-G expression if it recognised a common epitope on a HLA Class I molecule on HLA-G, which, in terms of its structure, may well have been the case. The results seen here contrast with the study by Schust et al. (1998), which appear to demonstrate the efficacy of HLA-G, HLA-Cw*0401 expressing JEG 3 cells at resisting rapid degradation associated with the presence of US11. There may be a difference in mechanisms between an immortalised cell line and primary cells that may account for this, but this would need to be investigated thoroughly. Again, due to the ambitious nature of this project, this was the limit of testing achieved for this strategy, but good progress has been made in investigating this aspect of universal T cell development.

Finally, TCR knockdown was investigated by numerous strategies, including ER retention, blocking of TCR/CD3 signalling and by genome-editing molecules. Unfortunately, all of these strategies, except for the TALEN molecules, failed to be successful at achieving TCR knockdown. Initially, the ER/Golgi retention strategy looked to have been working to some extent. On further evaluation, it seems as though the retention is not taking place despite the OKT3 and BMA031 scFvs binding to the TCR. One aspect of this that may be worth further investigation is the blocking of the TCR by the aTCR antibodies, which could effectively block TCR signalling. Another possibility could be to express these aTCR antibodies on the cell surface, protruded from the cell surface by a long flexible linker, which may allow the antibody to bind and block the TCR in an autocrine or paracrine manner. This may also be a way of blocking other cell surface signalling molecules, and it is worth investigating strategies such as adding a linker to an antibody in order to allow it to loop back and block ligand binding or signalling transduction of cell surface molecules.

Trying to target other aspects of the TCR signalling complex, such as CD3, by the ER retention strategy was also ineffective. With the CD3 strategy, the CD3 components may get retained too early in the assembly to be incorporated with the TCR, although one study does suggest that COPI retention in TCR assembly is caused by a region of the CD3 epsilon component, which is masked once assembly is complete (Mallabiabarrena et al. 1995). Despite slight effects seen by the CD3ε retention constructs, it seems as though this strategy did not work either. Subsequently, introducing a null CD3 construct, which lacked the individual component endodomains was tried in order to prevent TCR signalling. This was unsuccessful when analysed by intracellular cytokine staining. Many of these approaches may have been partially successful, but due to the high levels of TCR expression on the T cell, may have had a comparably negligible effect.

The direct approach was to use TALENs to target the TCR at the genomic level. Although the efficiency of this process can vary, when compared to transgene expression methods, it is very effective at disrupting a gene locus. TALENs obtained from Cellectis Bioresearch were successful at achieving

TCR knockdown. This targeted the TCRα constant gene, resulting in a reduction of TCR expression. For the electroporations successfully achieved, partial knockdown was shown to be present. This may not have been complete knockdown as the knockdown of TCR would result in a loss of signal activation and the subsequent loss of some survival signals, leading to a reduction in TCR^{lo} cell viability. In the timeframe of this project, unfortunately it was not possible to fully optimise the protocol needed in order to achieve repeatable and reliable electroporation that resulted in consistent levels of TCR knockdown, nor was it possible to develop a protocol that allowed for the selection of the TCR^{lo} T cells using the Miltenyi MACS system. Subsequently it was not possible to elucidate the functional effect of the TCR knockdown that was achieved.

Alongside the TCR knockdown, it was also shown to be possible to express a CAR in the T cells, which would re-direct the T cells that had lost TCR expression. In this way, it may be possible to prevent the unwanted GvHD effect, and also to target the T cells to a target of choice. The CAR would also provide the survival signals necessary for the T cells to survive and persist *in vitro* and *in vivo*. The CAR was able to be expressed in a small proportion of the TCR^{lo} cells, and the CAR was combined with the RQR8 sort-suicide gene that could allow for selection of modified T cells once a protocol has been fully developed.

CARs have now progressed to the third generation, with intracellular signalling domains becoming more robust in their activities, such as 4-1BBL, CD28 and OX40. Currently in the Pule group, the CD28 molecule is used as the endodomain, although a recent study has shown that using the 4-1BBL in the endodomain improves persistence of transduced cells *in vivo* (Song et al. 2011), so this could be investigated further. As well as the endodomains, the CAR work could also look into the type of viral vector that could be used in a potential clinical setting. So far, a retroviral packaging cassette has been used, but the ability of lentivirus to transduce quiescent T cells may be preferential, as stimulated T cells will terminally differentiate and therefore persist less *in vivo*. The envelope used to make the lentivirus could also be investigated as a recent study suggested that the measles envelope proteins haemagglutinin

(H) and fusion (F) are more efficient at quiescent T cell transduction than the commonly used VSVg envelope (Frecha et al. 2008).

6.2 Sort-suicide gene

The RQR8 sort-suicide gene developed by Brian Philip has also been incorporated into some of the above strategies, including the HLA class I knockdown and the introduction of a CAR to the T cells. In this study, RQR8 has been shown to be useful in cell sorting transduced from non-transduced populations, and in other work (Philip et al. 2014), has been demonstrated to be susceptible to Rituximab in *in vitro* and *in vivo* models. This has the dual function of being able to allow for clinical grade sorting of transduced cells, creating a homogenous population of therapeutic cells. The suicide gene also allows for rapid depletion of the therapeutic cells in case of a serious adverse event, such as the ones previously discussed. Depletion of therapeutic cells would prevent any further stimulation of the recipient's immune system and may reduce the effects and consequences of severe adverse events.

This study has included the RQR8 sort-suicide gene in combination other strategies, but the only function investigated in this study is the clinical grade sorting, which has proven to be effective. Analysis of efficacy as a suicide system would be needed for this project in order to determine which cancers would be amenable to this system. Currently, the suicide gene is a minimal CD20 epitope, recognised by the monoclonal antibody Rituximab. Rituximab recognises CD20, which is expressed on B cells, therefore use of this epitope as the suicide system would result in B cell depletion in the patient. It also prevents use of the therapeutic cells in any patient with B cell cancers and any patient already receiving Rituximab treatment. If it were possible to interchange the suicide gene epitope depending on the disease being treated, this would be a huge advantage over other suicide systems.

6.3 Additional obstacles of adoptive immunotherapy to overcome

Adoptive immunotherapy is a vast area, and as it involves manipulating the immune system, there are many facets of this subject that need to be investigated. This study investigates some of them, but there are others that have been omitted due to practical and time constraints. Other aspects to consider include the role of minor histocompatibility antigens (mHags), persistence of therapeutic cells, *ex vivo* expansion of therapeutic cells, immunogenicity of transgenes and the functionality of this whole approach in solid tumours.

6.3.1 Minor histocompatibility antigens

During allograft transplantations, recipients and donors have their HLA types matched in order to reduce the risk of allograft rejections as much as possible. Despite this, allograft rejection occurs even when donors and recipients are matched by their six major HLA alleles. One of the reasons for this is mHags. mHags are disparities in molecules outside of the MHC antigens, and when expressed on recipient cells and not donor cells, results in the donor T cells being stimulated by these mHags, leading to GvHD. The two main categories of mHags are sex-linked and non-sex-linked. The sex-linked mHags are ones that are expressed on the Y chromosome in males, and not in females. One study, by Miklos et al. (2005), looked at this set of mHags on the Y chromosome (H-Y). Each of these H-Y genes has an X chromosome homolog, which is found to be between 91-99% identical at the amino acid level (Lahn & Page 1997). Males develop tolerance to these; female T cells are capable of being activated by H-Y derived antigens following transplantation into male recipients (Wang et al. 1995). The study by Miklos et al. (2005) found that male patients with female donors were found to have a significantly greater frequency of antibody response that male patients with male donors. This antibody response has also been further characterised and been shown to be

high-titre and potent due to the combined response of both B and T cells to the H-Y antigens (Zorn et al. 2004).

One of the most commonly studied mHags involved in GvHD is HA-1. This mHag is recognised by HLA-A*0201 restricted CTLs and is present in around 69% of the HLA-A*0201-positive population (den Haan et al. 1998). Data from a previous study has shown a significant correlation between the presence of a HA-1 mHag mismatch and the incidence of GvHD in adult recipients of bone marrow, from genotypically HLA-identical donors. Whenever a HA-1 positive recipient received an allograft from a HA-1 negative donor, grade II or higher GvHD developed (Goulmy et al. 1996). Clearly, this needs to be taken into consideration in adoptive immunotherapy and this project, as, although HLA Class I has been knocked down, at the moment, it would be unfeasible to remove all of the HLA and mHags from the cell surface. Matching donors with recipients needs to be based on more than the traditional HLA major allele matching, although matching will never be perfect, even between siblings.

6.3.2 Persistence of therapeutic cells

Persistence of therapeutic cells is a key factor that needs to be taken into consideration. If cells do not persist for long enough, then the chances of tumour elimination are lowered and there is a higher chance of relapse. Whilst CAR therapies have been shown to be successful in treating malignancies in certain settings, poor persistence of therapeutic cells has caused poor clinical trial results. A phase I clinical trial for the treatment of metastatic ovarian melanoma using CARs directed against α–folate receptor (aFR) did not cause tumour regression due to poor persistence of the CAR T cells, and the lack of localization to the tumour (Kershaw et al. 2006). One way of improving persistence has been to introduce CARs into antigen-specific T cells against viral infections including EBV and CMV. These EBV or CMV-reactive T cells will be stimulated by any latent infection in the recipient, allowing for another method of co-stimulation *in vivo* (Savoldo et al. 2007; Di Stasi et al. 2009). One way of using an aspect of this project could be to create a third party bank of

HLA Class I negative EBV-CTLs which could be used in PTLD. These cells would not cause GvHD as they would be EBV-specific (Haque et al. 2007). By introducing resistance to Tacrolimus through the use of a calcineurin mutant, these EBV-CTLs could also be used as a therapy for someone undergoing immunosuppressive therapy (Brewin et al. 2009).

Enhanced *in vivo* persistence has been seen when therapeutic cells are expressing high levels of co-stimulatory receptors, suggesting that this is required for improved persistence (Powell et al. 2005; Zhou et al. 2005). As a result, subsequent generations of CARs have been designed with additional co-stimulatory domains, including CD28 intracellular domain, OX40 and 4-1BB. This results in CAR stimulation being able to enhance cytokine secretion and anti-tumour efficacy (Carpenito et al. 2009; Imai et al. 2004; Moeller et al. 2004). A study was carried out using various combinations of intracellular signalling domains for a CAR, where CD28, 41BB and CD3ζ were used. The CAR containing all three domains was found to be the strongest at promoting cytokine release, in vivo persistence and tumour elimination. Upon CAR stimulation, the strongest activation signals were obtained alongside the least induction of apoptosis in the therapeutic cells (Zhong et al. 2010; Kowolik et al. 2006). Therefore the design of the intracellular CAR signalling domains needs to be based on the best combination in order to elicit the optimal activation signals, driving the best tumour clearance possible and maintaining persistence *in vivo*. This can also be enhanced by performing codon optimisation to humanise any non-human domains to ensure immunogenicity risks are kept to a minimum.

6.3.3 Ex vivo expansion of therapeutic cells

Related to the obstacle of persistence, the *ex vivo* manipulation of the therapeutic cells needs to be determined also. When transducing T cells with viral supernatant containing transgenes, T cell stimulation needs to be used in order to stimulate proliferation, allowing for successful uptake of the relevant transgenes. Transduction efficiency needs to be as high as possible in order

to retain as many viable modified T cells as possible; therefore the stimulation protocol needs to be optimised.

Aspects to take into consideration include method of stimulation, duration of stimulation and type of viral preparation used in transduction. There are various protocols that have been used in the stimulation of T cells from PBMCs. Examples include performing cell selections to purify the T cells from PBMCs before providing cytokine stimulation, or adding cytokines that will stimulate T cells specifically from PBMCs. Both of these methods are then followed by transduction using viral supernatant containing the transgenes of interest. Currently, for autologous adoptive immunotherapy, it takes a few weeks for cells to be extracted, expanded, modified and prepared before they can be reinfused to the patient. In an allogeneic setting, it would be possible to have universal T cells ready to go, 'off the shelf', with the only delay being in the preparation of the cells for infusion, for example, thawing the cells from cryogenic storage.

Instead of using soluble cytokines to stimulate T cells, recent research has focused on the use of artificial antigen presenting cells (aAPCs) as the method of T cell stimulation. Magnetic beads coated with anti-CD3 and anti-CD28 antibodies were initially tested, which were found to stimulate long-term growth of $CD4^+$ T cells, but were not found to be able to induce long-term growth of $CD8^+$ T cells (Levine et al. 1997; Deeths et al. 1999). Co-stimulation was added by generating a K562 cell line that expressed CD32 and 41BB ligand (41BBL). These cells were also coated with anti-CD3 and anti-CD28 antibodies, and this was found to enable the long-term growth of CTLs (Maus et al. 2002).

The subset(s) of T cells being manipulated also needs to be taken into account. $CD4^+$ T cells are most commonly involved in the development of memory, and therefore are less efficient at inducing cell death. $CD8^+$ T cells are very adept at inducing cell death and eliminating target cells, but are less capable when it comes to developing a memory response. A balance of the two is needed if prolonged immune response to a particular cancer antigen is

required, so that the initial tumour can be targeted by CTLs, and so that persistence is maintained through the development of $CD4^+$ memory.

The differentiation of the T cells can also have an impact on persistence. If T cells have been kept in *ex vivo* culture for too long, then they may become terminally differentiated, resulting in a reduction in proliferative capability, and a subsequent reduction in long-term *in vivo* persistence when transferred into the patient. The more mature and differentiated the therapeutic cells are, the less they will be able to proliferate, and therefore persistence will be shorter. It has been shown that naïve or central memory T cells persist and engraft to a greater extent than terminally differentiated T cells (Berger et al. 2008; Hinrichs et al. 2009; Klebanoff et al. 2011). When transducing T cells, the type of viral supernatant affects how much stimulation the T cells receive during *ex vivo* manipulation. When using retrovirus, cells need to be actively proliferating in order to retrovirus to be taken up and for successful integration into the genome. Therefore, cytokine stimulation is required for efficient transduction. Retrovirus also tends to be associated with very low or undetectable levels of site-directed mutagenesis (Newrzela et al. 2008). Lentivirus is able to integrate into the host cell genome of quiescent cells, which provides an advantage if the aim is to minimise the amount of cell stimulation provided *ex vivo* (Cooray et al. 2012; Dufait et al. 2012). The drawback with lentivirus is that it is harder to achieve high levels of gene transfer, with typical ranges in clinical trials being between 5-23%, although protocol modifications are investigating whether the use of cytokines might improve this (Kalos et al. 2011; Gilham et al. 2012).

6.3.4 Functionality of therapeutic cells in solid tumours

Currently, CAR^+ T cells have been used in lymphomas, leukaemias and melanomas with clear effects. The next area of development of T cell therapy is in targeting solid tumours. The main obstacle here is finding a target antigen that is stably and exclusively expressed on tumour cells. Initial studies for CAR^+ T cells targeting carbonic anhydrase IX (CAIX) and aFR proved to be

unsuccessful in producing an effective outcome (Kershaw et al. 2006; Lamers et al. 2006).

An example of a solid tumour target is GD2, a disialoganglioside that is highly overexpressed in melanoma, neuroblastoma and Ewing sarcoma (Kailayangiri et al. 2012; Han et al. 2013). An additional obstacle with solid tumours is the reduction in vasculature compared to haematological cancers, which are present in the circulation and bone marrow niche. $1cm^3$ of solid tumour can contain 10^9 cells, therefore high numbers of CAR^+ T cells need to home to the site in order to elicit an effective response. CAR^+ T cells also need to persist and function in this immunosuppressive environment, and a lack of co-stimulatory molecule expression makes this a difficult issue to solve (Rossig 2013; Pedrazzoli et al. 2012).

6.4 Further work

There is clearly plenty of remaining work to be done on this project – this thesis investigated the beginnings of several aspects needed to develop a universal T cell. Work remaining on these aspects and other aspects not yet investigated are discussed in the following sections.

6.4.1 Validation of TCR knockdown

The work done so far on knockdown of the TCR has resulted in the demonstration of cell surface knockdown in T cells caused by TALEN molecules directed against the TCR α chain. What has yet to be demonstrated is the effect that this has on the T cell function. Several key checkpoints need to be passed in order to determine whether this strategy is one that can be pursued further.

The reason for investigation TCR knockdown is in order to prevent or reduce GvHD induced by donor therapeutic cells when given to the recipient.

Therefore, these TCR^{lo} T cells need to show a reduction in GvHD in an allogeneic setting. It has also been shown that it is difficult to maintain cell viability of the TCR^{lo} T cells, as keeping them in culture for a short amount of time results in large amounts of cell death when analysed by FACS (see Chapter Five). This could be due to a lack of survival signals that are normally received through the TCR, due to other cells, such as NK cells in the culture targeting the TCR^{lo} T cells for cell death, or a combination of both.

It is unlikely that it would be possible to demonstrate a reduction in GvHD if using just TCR^{lo} T cells against an allogeneic donor. Simultaneous, or subsequent introduction of a CAR is required in order to maintain cell viability and allow for a demonstration of a lack of GvHD. Introducing a CAR, though requires the use of a target cell line that expresses the CAR target, and therefore means that an assay needs to be developed to assess a reduction in GvHD and the efficacy of the CAR against its target. One way to demonstrate this *in vitro* may be to use an engineered SupT1 cell line that expresses the CD19 molecule. In Dr Pule's research group, this cell line already exists to validate CARs directed against CD19 for use in B cell malignancies. TCR^{lo} aCD19-CAR^{+} T cells could be used against various cell lines, including CD19-SupT1s to demonstrate CAR efficacy against the CD19 target, and also to demonstrate a lack of activity against the unmodified SupT1 cell line. This could be done in the form of a ^{51}Cr release assay, and unmodified T cells could be used as a positive control to demonstrate the usual levels of GvHD in this assay. Cell viability would also be assessed in order to determine if the CAR molecule was able to provide sufficient survival signalling for the TCR^{lo} CAR^{+} T cells to persist over extended periods of time.

A study performed since the conclusion of these experiments has demonstrated effective knockdown of the TCR by the use of TALENs directed against either the TCRα or the TCRβ chains. In particular, one TALEN directed against the TCR-α2, in three different donors and six experiments, increased the proportion of TCR-negative cells from 5.6% (+/-1.1%) in mock-transfected cells to 58.5% (+/-15%) in cells transfected with the TALEN-TCRα2 mRNA. This was further supported by sequencing of 17 clones, which demonstrated genomic deletions in the TCR-α2 locus in nine of these clones (53%). These

TCR negative cells then had a transgenic TCR introduced and were shown to be functional, secreting a significantly higher amount of IL-2 and IFNγ when stimulated with APCs bearing a Flu tetramer (Berdien et al. 2014). This demonstrates that it is possible to knockdown the endogenous TCR and to then subsequently redirect a cell's specificity with a transgenic TCR. This study, amongst others, would form the basis for repeat experiments studying the effect of the TALENs used in this thesis. An aspect of the study by Berdien et al. (2014) that would need to be streamlined would be the protocol. In the study, the protocol involved TCRα knockdown, followed by the enrichment of TCR negative cells by CD3 depletion. These TCR negative cells were then transduced with the transgenic TCRα chain. Following this, TCR positive cells were selected by FACS sorting before being treated with TALENs against the TCRβ locus. TCR negative cells were enriched by a further CD3 depletion before these were transduced with the transgenic TCRβ chain. This created a population of T cells that were negative for endogenous TCR and positive for the transgenic TCR. A similar strategy was used by (Provasi et al. 2012) using ZFNs instead. It is a complex and lengthy process that could be improved with the use of CARs as the transgene instead. This would allow for knockdown of the TCR by targeting one chain, followed by a sort and transduction with the CAR transgene. There would not be the risk of the endogenous TCR pairing with the transgenic TCR that had to be circumvented by the above study, and would also mean that only one electroporation, depletion and transduction step would be needed; a protocol that could be completed in less than two weeks.

Another approach that has been recently developed is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR Associated (Cas) systems. The CRISPR system in bacteria relies on the acquisition of foreign DNA fragments into CRISPR loci in the bacterial genome, which then undergo transcription and processing into CRISPR repeat-spacer arrays into short CRISPR RNAs (crRNAs). The crRNAs then anneal and become a trans-activating crRNA (tracrRNA), which direct sequence-specific silencing of foreign nucleic acid by the mechanism of the associated Cas proteins (Hwang et al. 2013). Synthetic guide RNAs (gRNA) can be adapted from the system in

order to guide a nuclease to a specific DNA site and then cause a DSB. To date, some of the applications for this technology has involved screening for disease-related genes and generation of knockout models (Shalem et al. 2014; Yang et al. 2014; Gaj et al. 2013; Li et al. 2013; Xiao et al. 2013). Clearly, the use of CRISPR in TCR knockdown is a strategy that needs investigating, as there is currently no literature published in this area.

6.4.2 Functional validation of HLA-G transgene

As with the work on TCR knockdown, the functionality of HLA-G has not been evaluated yet; therefore the next steps need to be the functional testing in MLRs and to test NK cell activity in response to HLA-G expressing cells. The K562 cell line is HLA Class I negative, as discussed earlier, therefore introducing the HLA-G transgene to these cells would allow for testing of the functional effect of HLA-G. A proliferation assay or a cytokine release assay would be suitable for assessing this.

Although HLA-G is a non-classical HLA molecule, it does share similarities with HLA Class I molecules, and this was shown when attempting to co-transduce the SupT1 cell line with the HLA Class I knockdown and HLA-G transgenes (**Figure 40**). When attempting to do this, the level of HLA-G expression was quite low, suggesting that the ubiquitination caused by the US11 transgene might also target HLA-G. Therefore, in case it proves to be unviable to co-express the HLA Class I knockdown and HLA-G transgenes in the same cell, alternative strategies need to be investigated, such as HLA-E, HLA-F and UL142. UL142 is a HCMV derived protein that is heavily glycosylated and is expressed during late stage infection. Knockdown of UL142 by siRNA in HCMV-infected cells resulted in an increase in vulnerability to NK cell killing, suggesting that this molecule has a role in inhibition of NK cell activation (Wills et al. 2005). If HLA-E and HLA-F also have structural similarities to HLA Class I, then UL142 may be a viable alternative for inhibition of NK cell lysis. Another option may be to use a genomic-targeting strategy, as was demonstrated by Torikai et al. (2013) in the use of ZFNs.

6.4.3 Combination of strategies

The key to this project is the ability to combine the strategies that have been successfully tested. The initial aim was to have one viral cassette that contained all of the transgenes, separated by the FMD 2A self-cleaving sequence. This would have allowed for one transduction to be performed, followed by cell recovery and MACS-based cell sorting, resulting in a homogenous T cell product that could be used for adoptive immunotherapy. As this project has progressed, a slightly different approach has been developed, as not all strategies were successful at a protein level, so a single viral cassette is no longer possible. If the strategies tested were to be combined, then a viral cassette could be developed that contained the HLA Class I knockdown, HLA-G, CAR and sort-suicide transgenes. For the TCR knockdown, this would need to be done through the electroporation of RNA into the T cells; therefore a two-step protocol looks more likely.

During the project, it has been possible to combine some of the strategies together and expression of them has been demonstrated, although functionality in these combinations has not necessarily been shown. HLA Class I knockdown strategies were combined with the RQR8 sort-suicide gene (**Figure 25** and **Figure 27**), and the CAR was also combined with the RQR8 sort-suicide gene (**Figure 71**). For these ‘double’ transgene constructs, expression of both elements was demonstrated, as well as the ability to use the RQR8 sort-suicide gene to sort the transduced cells to high levels of homogeneity. Although not in one viral cassette, it has been possible to co-express the RQR8-2A-HLAko constructs alongside the HLA-G transgene, showing promise that these three have the potential to be combined. With more time to work on this project, the development of a construct that contained all these five transgenes would be a high priority once functional work had been performed on each of the strategies individually. For the RQR8 sort-suicide gene, this has formed the basis of another project in the research group, which has clearly demonstrated that both the sorting and suicide

mechanisms are functional and effective in murine models (Philip et al. 2014). Once the strategies have been proved to be functional *in vitro*, individually and combined, the next step would be to test them *in vivo*, in a model organism, with the preferred model being a humanised SCID mouse model. This would allow for an allogeneic immune system to be developed in the mouse before the addition of tumour cells, followed by the therapeutic T cells, and would allow for a model that is humanised, preventing the need to optimise or modify transgenes for murine expression before testing.

For each of the strategies, the following work needs to be done before they can be combined into a single viral construct. For the HLA Class I knockdown, the impact of the HLA knockdown needs to be fully quantified before introducing HLA-G to the HLA Class I negative T cells. MLRs can be performed with the HLA Class I negative T cells to determine the level of NK cell killing induced by the therapeutic T cells. This can be measured by cell viability analysis. Once this has been established, HLA Class I negative, HLA-G-RQR8 positive T cells can then be used in an MLR with NK cells to demonstrate any reduction in NK cell killing activity. Further MLRs will also be used to test activation of the T cell effector population, which will have been transduced with the HLA knockdown and HLA-G transgenes, against target cells, to determine if a reduced response occurs as a result of the knockdown. NK cells will be depleted from these MLRs as they may skew results by killing the HLA knockdown cells, preventing quantification of T cell activation by the effector population.

The RQR8-CAR construct will need to be tested in a ^{51}Cr release assay to determine activity against the CAR targets. The effector cells will be sorted using the RQR8 sort-suicide gene before using them in the assay. The assay can also be performed in the presence, or absence of Rituximab in order to demonstrate lack of activity when the RQR8 suicide system is activated, and to demonstrate the successful killing of RQR8-CAR T cells *in vitro*. The TALENs directed against the TCR can then be incorporated into these assays, with there being an additional observation for the presence or absence of GvHD. RQR8-CAR^{+}, TCR^{lo} T cells can finally be tested in an allogeneic setting to demonstrate a lack of GvHD against the mis-matched cells, with activity only

present against CAR-targeted cells. Once the RQR8-HLAko-HLA-G construct and the RQR8-CAR construct have both been tested and shown to be fully functional *in vitro*, they will be combined to create one viral cassette.

6.4.4 CD52 knockdown

One additional aspect to this project was investigating the possibility of CD52 knockdown from T cells. CD52 is a molecule expressed on lymphocyte cells and Campath-1H is a mAb used in the treatment of CLL amongst other malignancies (Domagała & Kurpisz 2001). It eradicates cells expressing CD52, both donor and recipient, treating the malignancy but leaves the patient vulnerable to infection (Ratzinger et al. 2003). If a patient, who is already receiving Campath-1H, is given therapeutic T cells, then they will be depleted by the Campath-1H treatment. By knocking down CD52 from the therapeutic cells, therapeutic T cells should persist *in vivo*.

Multiple antibodies were found in a patent that bound to the twelve residue CD52 epitope (Roberts et al. 2010). Two of these antibodies were selected based on their recorded binding to the CD52 epitope – 7F11.1.9.7 recognises the first five residues of glycosylated CD52; 9D9.A2 recognises residues three to seven of the CD52 epitope, with no preferential binding to glycosylated or non-glycosylated CD52. Both of these antibodies were cloned, tested for binding affinity and then an ER retention sequence was attached to the C-terminus. When retroviral supernatant was transduced into T cells, these two antibodies were unsuccessful at achieving CD52 knockdown. CD52 is normally expressed on the cell surface by a GPI anchor, therefore one other strategy tried was to attach the antibodies to a GPI anchor and aim to disrupt CD52 folding and assembly before reaching the cell surface. Again, this was unsuccessful.

Despite this, there are TALENs targeted to CD52 that are able to achieve CD52 knockdown (Cellectis), so this now becomes a viable strategy to develop

therapeutic T cells that have a CAR, as well as being both TCR and CD52 negative. These T cells would be able to be used to treat someone undergoing Campath-1H therapy, and could result in improved recovery rates and tumour clearance.

6.5 Final conclusions

Since beginning this project, there have been several studies done that have also investigated the development of T cells in adoptive immunotherapy, which need to be highlighted to show other approaches and strategies used to achieve similar aims to the ones that have been investigated here. A study by Torikai et al. (2012) looked at developing T cells that expressed an aCD19-CAR and had endogenous TCR expression knocked down. The *Sleeping-Beauty* (SB) transposon/transposase system was used to introduce ZFNs that targeted the TCR α or β chains. The aCD19-CAR was introduced using the SB system and the ZFNs were introduced by electroporation of RNA. Electroporation efficiency was up to 37%, and the $CD3^-$ T cells were enriched by multiple rounds of $CD3^+$ depletion, resulting in populations of over 99% $CD3^-$ T cells. The engineered cells were demonstrated to have efficacy against CD19 expressing targets whilst remaining unresponsive to TCR stimulation. Propagation of these cells was also possible by using $CD19^+$ aAPCs. This study was further built on by Torikai et al. (2013), which investigated the elimination of HLA Class I from the surface of T cells. Again, this was done using ZFNs, and resulted in an elimination of 52% HLA-A from the modified T cells. These $HLA\text{-}A^-$ CAR^+ T cells were enriched to ~94% purity and could evade lysis by HLA-restricted CTLs, as well as maintaining their CAR activity. Inhibition of NK cell killing was also shown by expression of the non-classical HLA molecules, HLA-E and HLA-G, on HLA Class I negative 721.221 cells. These two studies show that there are major steps being made towards achieving the aim of developing a universal T cell for use in adoptive immunotherapy. The key difference between this project and these two studies is the method of achieving this aim. This project has aimed to combine as

many strategies as possible into one viral cassette, thus reducing the level of manipulation and complex protocol optimisation that is needed when using genome-editing nucleases such as ZFNs and TALENs. If the above two studies were to be combined, it would require multiple steps of electroporation, as well as multiple rounds of cell selections in order to obtain the desired cell population.

Creation of a third party T cell would be a huge step forward as it would significantly reduce the costs and risks involved in adoptive cell therapy. HLA knockdown and TCR knockdown would reduce the risk of the alloreactions currently hindering allogeneic T cell therapies. Using a CAR allows re-direction of the T cell and a molecule such as HLA-G could ensure survival of the T cell by avoidance of NK cell killing in the recipient. Finally, a sort-suicide gene would allow clinical grade sorting of transduced cells and provides a mechanism to delete therapeutic T cells in the case of toxicity and/or adverse reactions. Combining these elements would provide the first steps towards the creation of a universal T cell and significantly reduce the time required for T cell engineering and provide an ‘off-the-shelf’ therapeutic for various malignancies. This project has investigated the first steps towards achieving this aim, and provides a body of work to help direct future developments.

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